THE ROLE OF TCR GAMMA/DELTA T-CELLS IN THE GUT EPITHELIAL COMPARTMENT

by

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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

May 2017

ABSTRACT

T cells expressing the γδ TCR continue to be an enigmatic immune cell subset. To study the role of γδ T cells in the functioning of the gut epithelial barrier we took a whole-genomics microarray approach to examine pathways in the GI tract that are effected by the absence of γδ T cells. We also examine the effects of the absence of γδ cells on the type of immune response elicited, as well as, the timing of the immune response to *Salmonella enterica typhimurium*. A number of the pathways dysregulated in the absence of γδ T cells point to the pathways involving gut epithelia maintenance, regulation, and functionality.

Based on the genomic analysis, we investigated the effect of the absence of γδ T-cells on the functioning of the gut epithelia. Using a series of FITC-Dextran species with varying molecular weights (4kD to 100kD) we observed an increase in permeability to low molecular weight species in mice lacking γδ T cells. This suggests an impairment of tight junction formation and/or structure in the absence of TCR γδ T cells. In the setting of *S. typhimurium* infection we observed an increase in barrier integrity loss in infected TCR γδ T cell deficient mice versus their wild-type counterparts. We also examined the structure of the epithelial layers in the jejunum of TCR γδ T cell deficient mice versus wild-type mice using transmission electron microscopy (TEM). The TEM study shows a decrease in tight junction formation and significant structural differences in TCR γδ T cell deficient compared to normal WT tissue. Collectively these studies reveal a novel role for TCR γδ T cells in maintaining the functional integrity of the intestinal epithelial barrier.

ACKNOWLEDGEMENTS

First and foremost I would like to thank the Lord for giving me the vision and strength to see this endeavor through. Without Him, none of this would, or could, be possible.

I want to especially acknowledge my mother, Mrs. Debra E. Barrett, who is not only my beloved mother but a spiritual sister, a confidante, an encourager, and my best friend. She held onto to my dream when I alone did not have the strength to do so. This is dedicated to her. She has always been my first and very best teacher. Words will never fully express my gratitude. Thank you, Ma!

To my grandmother, family, and friends whose words and love encouraged me along this journey. Thank you.

I also want extend my appreciation to my advisor, Dr. Mark J. Soloski, whose patience and guidance were tremendous gifts to me. He will never be forgotten.

Lastly, I want to thank my committee members. In particular, I would like to give special thanks to Drs. Christopher Cheadle and Alan Scott whose insights and ideas were invaluable to this project.

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INTRODUCTION

T-cells have two main cell lineages clonotypically expressing either TCRαβ or TCR γδ receptors. The functions of the TCR γδ receptor T-cells are less well understood than their TCRαβ counterparts. TCR γδ T-cells represent approximately only 2% of total T cells found in lymphoid tissue.[1] However they are enriched in the mucosal and epithelial barrier of skin tissues, lungs, and gastro-intestinal tract (referred to in this text as the GI-tract or gut). Of the enriched locations, the γδ T-cells, are found most abundantly in the gut mucosa where they form an important component of a population of lymphocytes known as intraepithelial lymphocytes (IELs).[2] In the gut, TCR γδ T-cells reside within the epithelial barrier lining which includes the region from the sub-epithelial layer to the apical barrier of the intestinal lumen (Figure A). These TCR γδ T-cells and TCR αβ T-cells are commonly referred to as intraepithelial lymphocytes (IELs). IELs have been found in all examined vertebrate species implying a conserved, fundamental role.

In both human and mouse studies, TCR γδ T-cells have been shown to recognize stressed cells and to release effector molecules that promote wound healing.[3] The γδ TCR T cells that reside specifically in skin are termed dendritic epidermal T cells (DETC's) because of their location and because their morphology. DETC's are thought to "capture" unknown antigen expressed by wounded (or infection-induced wounded) keratinocytes using γδ T-cell receptors.[4] Activated DETC effector show that when activated by the presence of damaged keratinocytes, DETC produce a crucial keratinocyte growth factor for wound repair called fibroblast growth factor 7 (FGF-7).[5,6] This growth factor has been produced both *in vitro* and *in vivo*, which supports the hypothesis that DETC could play a pivotal role in the biological/biochemical pathways of

FIGURE A – THE INTESTINAL EPITHELIAL BARRIER

Figure A is a schematic of the epithelial barrier of the intestinal tract. Depicted are the apical barriers separating the intestinal cell from the interior of the tissue, the lumen; the basal side (baso) separating the tissue from the bloodstream; and the lateral boundaries that separate each intestinal cell. Resident immune cells are dendritic cells and thee intraepithelial lymphocyte γδ T-cells. The γδ T-cells shadow tight junction structures along the apical villi. The tight junction complexes are composed of its chief structural proteins occludens and claudins.

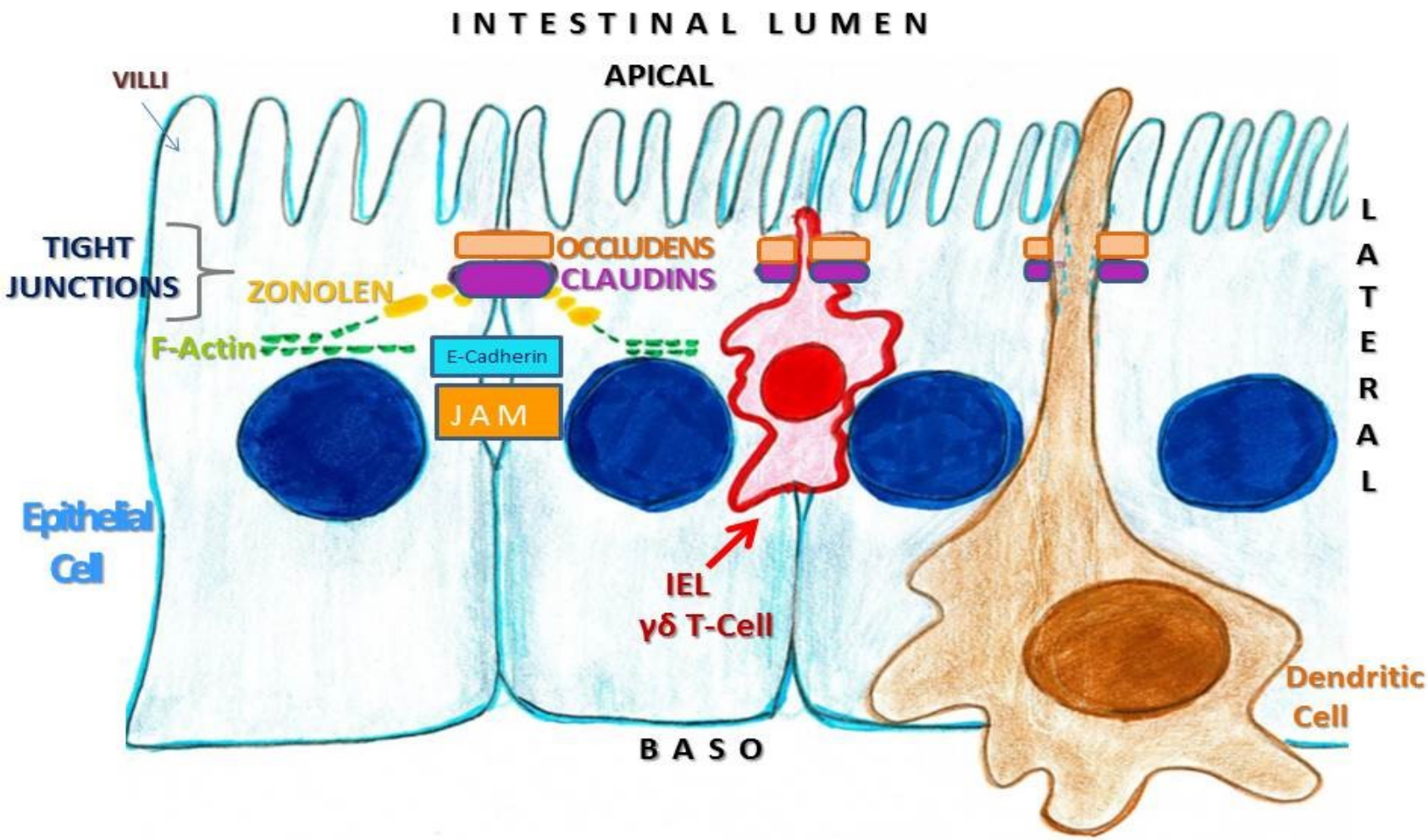


Figure A: The epithelial barrier of the intestinal tract.

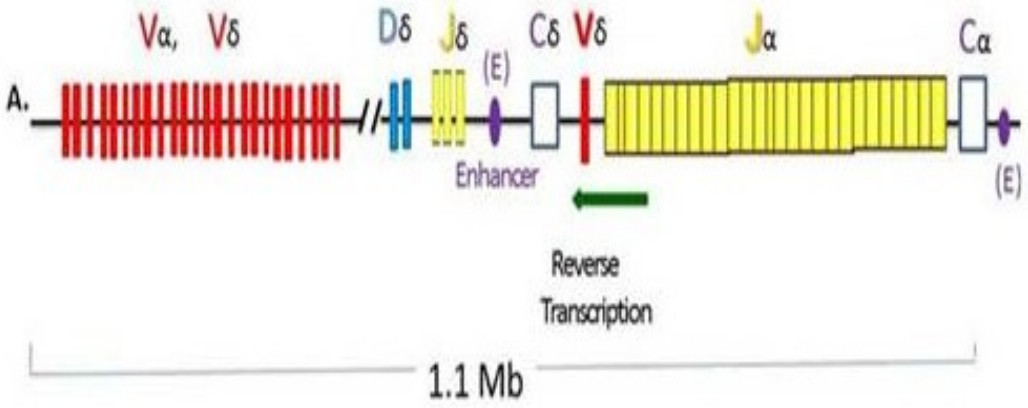
wound repair.[7] It has also been shown that keratinocyte proliferation is decreased and wound closure is slowed, respectively, in TCR γδ T-cell-deficient mice.[8] DETC's produce chemokines and cytokines as part of their effector functions in their immune response to injury or infection.[9] The control of inflammatory responses to the "biological insult" that occurs in epithelium wound injury may provide another pathway to establish homeostasis.

The extensive structural diversity of γδ TCR is generated through somatic recombination of gene segments termed variable (V), diversity (D) and joining (J) segments (Figure B).[10] γδ TCRs are non-covalently linked to a transduction complex composed of several CD3 subunits, which triggers intracellular signaling cascades and subsequent activation of T-cell effector functions following antigen recognition. Besides γ and δ TCR chains, which are by definition the most reliable markers allowing identification and isolation of γδ T cells, γδ T cells carry on their surface a large set of surface molecules whose expression can be detected by flow-cytometry using labelled monoclonal antibodies. TCR γδ T cells share markers with αβ T cells (e.g. CD2, CD3, CD7) and other hematopoietic cells. However γδ T cells do have unique markers not shared with other hematopoietic cells, of which, several are preferentially expressed by one or other T-cell subset. In rodents and humans, CD4 or CD8 co-receptors are found on a majority of mature αβ T cells but only on a fraction of γδ T cells derived from non-epithelial sites. CD8 γδ T cells have a higher population in the intestinal mucosa of most animals studied thus far.[11]

However, unlike CD8+ αβ T cells, which generally express heterodimeric CD8 co-receptors (having α^+ and β^+ subunits), most γδ T cells express homodimeric CD8

FIGURE B – THE γ AND δ GENE LOCI AND THE MECHANISM OF SOMATIC RECOMBINATION

T cell receptor loci are rearranged in the order delta to gamma. A. The germline organization of the T cell receptor δ locus embedded in the 1.1 Mb TCR α loci. The δ locus includes V (variable), D (diversity), J (joining), and C (constant) segments. The δ chain is synthesized by a rearrangement of the V segment to a D segment and to a J segment with the deletion of the intermediary DNA; the C segment is later joined by splicing at the RNA level. B. This region represents the germline organization of the TCR γ locus. The γ locus includes V (variable), J (joining), and C (constant) segments. The γ chain is synthesized by a recombination event at the DNA level joining a V segment to a J segment with a deletion of the intermediary DNA. The C segment is later joined by splicing at the RNA level. C. All gene segments between the V-D-J gene segments in the newly formed complex are deleted and the primary transcript is synthesized that incorporates the constant domain gene (V-D-J). mRNA transcription splices out any intervening sequence and allows translation of the full length protein for the TCR chain.


A.
Vα, Vδ
Dδ
Jδ
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Cδ
Vδ
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Cα
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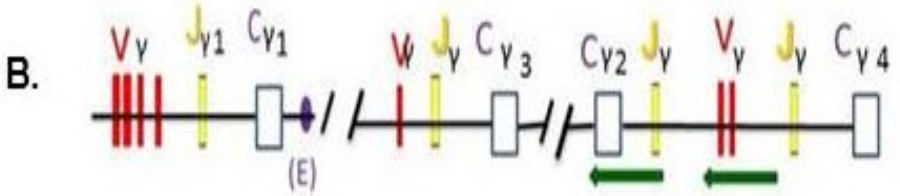
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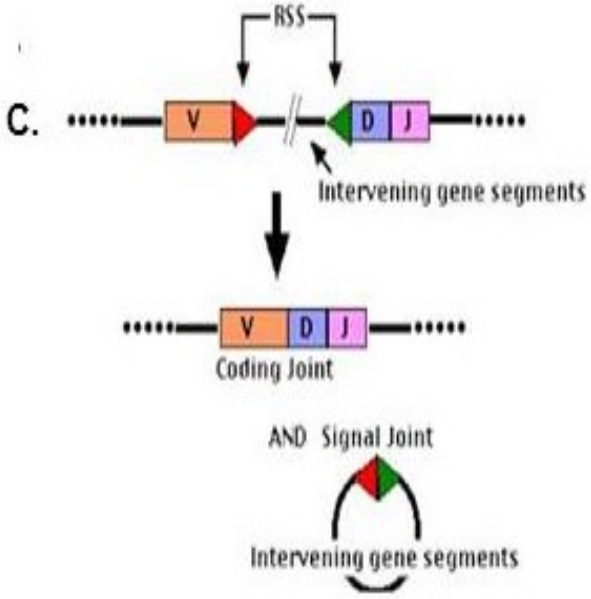
RSS
C.
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Intervening gene segments
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Coding Joint
AND Signal Joint
Intervening gene segments

α^+/α^+ molecules. There are several explanations for these phenotypic differences that are related to the distinct developmental features, antigen specificity and activation status of αβ and γδ T cells. In particular, the lack of both CD4 and CD8 co-receptors on most γδ T cells probably reflects their strong reactivity against major histocompatibility complex (MHC)-unrelated ligands.[12] Furthermore, the frequent expression of CD8 α^+/α^+ homodimers by intestinal intraepithelial γδ T cells is probably due to their chronic *in vivo* stimulation.[13,14] Expression of CD8 α^+/α^+ homodimers, unlike CD8 α^+/β^+ heterodimers, can be induced on various lymphoid cells, including γδ T cells and natural killer (NK) cells, following their *in vitro* activation.[15,16]

CD8 γδ T cells taken *ex-vivo* display several phenotypic and functional markers of pre-activated/memory T cells such as CD25 or CD45RO.[17,18] Unlike αβ T cells, γδ T cells frequently share several markers with NK cells, including the coactivator NKGD2 homodimer, the FcγRIII receptors CD16 and CD56.[19] Several inhibitory receptors binding to MHC class I (class 1), such as CD94/NKG2 heterodimers, are also frequently detected on blood NK and γδ T-cells but seldom found on αβ T-cells.[20,21] The expression of class 1 molecule receptors may enable γδ T-cells to regulate their activation through signals received from the immune synapse.[22,23] Also, as for NK cells, MHC class 1 recognizing receptors might help in controlling the inherent self-reactivity of some γδ T cells subsets by delivering inhibitory signals upon interaction with self MHC class I molecules.

One γδ T cell subset, the IL-17-producing γδ T cells, has recently been shown to have a major pathogenic role in autoimmune diseases.[24] γδ T cells, NKT, and other innate lymphoid cells have been shown to be major sources of IL-17 in host control of a

variety of bacterial, viral, and fungal infections.[25] Dysregulation of these innate-like lymphocytes can result in pathology in the form of experimental autoimmune encephalomyelitis (EAE) and other models of autoimmunity for MS and CNS.[26] IL-17-secreting γδ T cells are rapid and potent mediators of inflammation because they can be stimulated with cytokine stimulation alone. [27] γδ T cells can secrete IL-17 in response to IL-1, IL-18, and IL-23 without TCR engagement, promoting the induction of Th1 and Th17 cells during the development of EAE. [28,29,30] Studies have shown that dendritic cells can enhance the ability of IL-1- and IL-23-activated γδ T cells to promote IL-17 production by Th17 cells.[31] This suggests that γδ T-cell-derived IL-17 acts in a positive feedback loop involving dendritic cell activation leading to enhanced Th17 cell effector function during EAE.[32]

TCR γδ T-cells and Infection Response

TCR γδ T-cells have also been implicated in the response to infection. TCR γδ receptor T-cell expansion has been observed after infection with *Salmonella typhimurium* and TCR γδ receptor T-cell subsets have been implicated in the response to infection with *Listeria monocytogenes* and *Toxoplasma gondii*.[33,34,35,36] In the case of *S. typhimurium* infection of mice, *Salmonella* usually remains localized to the intestinal epithelium and the gut-associated lymphoid tissues where this organism causes severe enteritis. However, in the case of *S. typhimurium* infection of mice and *Salmonella typhi* infection of humans, *Salmonella* infection can become systemic with bacteria rapidly migrating to a several sites in the body, including the spleen and liver, where they can replicate in phagocytic cells.[37] T cells of the adaptive immune system play a central role in the

clearance of primary infections and in protection against subsequent challenge with related strains of *Salmonella*.[38] However, T cells that reside in the intestinal epithelium (IELs) are the first elements of the host T-cell compartment available to respond to *Salmonella* during its entry into the host by oral infection.

Recent reports have shown that depletion of cells during *S. typhimurium* oral infection in mice results in significant decrease in clearance of the bacterial pathogen in the intestine and other tissues, suggesting that IELs, particularly TCR γδ T-cells, have an important role in both the detection of pathogenic bacteria and eradication of infected epithelial cells.[39] Similarly, these data are also consistent with reports of reduced intestinal epithelial integrity in the absence of TCR γδ T-cells leading to an inability to restrict epithelial transmigration of either *Toxoplasma gondii* or *Salmonella typhimurium* in response to infection.[40]

The Gastro Intestinal Tract

The Small Intestine

The small intestine is the organ mainly responsible for the absorption of nutrients into blood.[41] In order for absorption to occur, food must be broken down into groups of smaller molecules that, in turn, must be broken down into molecules small enough to be diffused across the epithelium. Once within the small intestine, these molecules are exposed to pancreatic enzymes and bile, which enables digestion to molecules capable of being absorbed. The final stages of digestion occur on the surface of the small intestinal epithelium where molecules are selectively diffused across the intestinal epithelium. The net effect of passage through the small intestine is absorption of most of the water and

electrolytes (sodium, chloride, potassium) and almost all dietary organic molecules (including glucose, amino acids and fatty acids).

The tissues of the small intestines include the duodenum, jejunum, and ileum. Each tissue has a specific function in the small intestines' roles in digestion and absorption. The duodenum function is to chemically process chyme, or partially digested food that is secreted from the pancreas, liver, and gall bladder, and to carry the chemically digested food, or macromolecules, to the jejunum.

The jejunum is the midpoint of the small intestine and is the major site of digestion and absorption. The first place where nutrients are exchanged is the apical side of the jejunum. The apical side faces the lumen, or the interior of the intestines. The side of the lumen that meets the inner most intestinal wall is called the mucosa layer, which is covered with epithelial cells bearing projections called villi. In addition to these epithelial cells, the mucosa also contains secretory and endocrine cells. When nutrients have been broken down into their smallest units, they can be passed to this tissue, absorbed through the jejunal wall and actively transported into the blood stream where it is carried to the cells of the body through the circulatory system. The mature absorptive epithelial cells take up nutrients from the lumen and transport them into blood, fulfilling the basic function of the digestive system.[42]

The main reason that the jejunem tissue is of special interest to our study is because, in addition to being the main location of absorption in the body, the jejunum is the location where pathogen enters the hosts' system and, eventually, crosses the threshold into the circulatory system.[43] This development is the main reason for systemic

tissue failure that prolongs the disease state and immune response cycles that can further damage tissue

Our Hypothesis

The gut epithelial functions to provide a protective barrier from enteric pathogens as well as to facilitate the absorption and distribution of needed nutrients. These functions are accomplished by the concerted action of a number of interacting cell types including epithelial cells, stromal elements and immune cells. Our hypothesis is that dysfunction of any one of these cell types will have adverse effect on the capacity of the epithelial tissue to continue their key functions. In particular, we believe that cells of the immune system are an essential component for the functioning of the gut epithelia. Not only do they have a function in the initial host response to enteric pathogens, but are likely to function through the release of soluble mediators and/or cell-to-cell interactions in the maintenance of the integrity and function of the epithelium. We have used a whole-tissue genomic based approach to understand how the particular novel subset of intraepithelial lymphocytes, γδ T cells, contributes to the functioning of the gut epithelial barrier. Using mouse models positive for TCR γδ (WT strain) and negative for TCR γδ (knockout strain) in microarray analysis, we examined the genes and pathways of the GI tract that are affected by absence TCR γδ T-cells. As a secondary part of our analysis, we examined the effects of TCR γδ on the type of immune response, as well as, the timing of the immune response to *Salmonella enterica typhimurium*. One part of our method to examine host response to biological insult is to use the data gathered with the uninfected WT and KO for the TCR γδ T-cells as a baseline to examine the "normal" genetic footprint and pathways elucidated from the gene analysis. From these analyses, we could

understand the how the gene regulation altered before infection, and in the wake of the removal of the γδ T-cell population from the gut, affected the KO host pattern of gene regulation in response to infection.

Some of the pathways elucidated from this gene analysis helped us determine the *in vivo* models we would use to determine how the absence of TCR γδ T-cell population in the gut epithelia impacts function. Since many of these pathways are significantly upregulated with the absence of TCR γδ T-cells they may reveal novel components involved in gut epithelia maintenance, regulation, and functionality. We studied *in vivo* models of gut epithelial membrane permeability with a series of FITC-Dextran experiments with varying molecular weights. These results led us to challenge the gut epithelia with *S. typhimurium*, and observe if barrier function was impaired in the KO. Our goal is to examine if the absence of TCR γδ T-cells in the gut could impair barrier function and impact further systemic tissue damage to the host.

Finally we use transmission electron microscopy to study the structure and composition in the mucosal layer of the gut epithelium. Taking the results from the genomic data and the permeability as a basis for this series of experiments, we isolated jejunum tissue from WT and KO mice to focus on the tissue development of vital, functional structures such as tight junctions and boundaries of the apical and baso-lateral structure.

CHAPTER 1

The Impact of TCR γδ T-cells on the Normal Tissue of the Gastro-Intestinal Tract Using Whole Tissue Genomic Analysis and Phenotypic Expression Examination

INTRODUCTION

This part of our studies applies whole-tissue genomic analysis to intestinal tissues from normal mice (WT) and TCR □□ T-cell deficient mice (KO). RNA used was extracted from the jejunum, ileum, cecum, ascending and descending colons of the GI tract; and the liver. Data gathered from the tissue of WT and KO mice helped to identify genes and pathways influenced by TCR γδ T-cells. The jejunum, one of the eight tissues studied, is the primary focus of interest, although not the sole interest of this text. The highlighting of the uninfected, normal and KO tissues became important because we could identify the response of gene expression differences and deficiencies in the TCR γδ T-cell. This will become the baseline information as to response to infection. We will use this data to understand the response to Salmonella *enteria typhimurium* (*S. typhimurium*) infection in Chapter 2.

The hypothesis for this set of experiments is that the study of the mucosal epithelia of uninfected WT and KO mice will demonstrate that TCR γδ T-cells are an essential component for the normal functioning of the gut epithelia. Another part our hypothesis is that not only do γδ TCR T-cells have an integral role in the initial host response to enteric pathogens, but they also are likely to function, through the release of soluble mediators and/or cell-cell interactions, in the maintenance of the epithelium barrier. [44,45,46] The technique by which we examine the functioning TCR γδ T-cell functioning in the gut is specifically remove this cell population from other resident IELs. The effects of just removing TCR γδ T-cells in the gut epithelium are first studied at the transcriptional level using microarrays and gene set analysis. We reasoned that removing this cell population will allow us to examine its role in normal gut epithelial integrity

prior to infection and tissue damage. This source of experimental information helps to elucidate potential biological and biochemical pathways that shed understanding about the role of TCR γδ T-cells in the functioning of the gut mucosa. Specifically, we want to understand the structural architecture and environmental homeostatic maintenance of the tissue. We continue to examine the impact of these γδ T-cells on gut function at the phenotypic level by conducting *in vivo* membrane permeability tests on both mouse strains and by viewing tissue under the high powered magnification of transmission electron microscopy.

METHODS

Generation of the TCR γδ T-Cell Knockout Mice

The WT mice, which have been obtained from the mouse repository JAX® Laboratories, are one of the most popular research strains to study disease, the C57BL/6 strain (also known as C57BL/6J, BL6, or Black 6). These mice were inbred with male-female sibling breeding pairs and used as a genetic background strain for the KO, or Tcrd^{tm1Mom} in JAX® gene nomenclature. The *Tcrd*^{tm1Mom} target mutant strain was developed using a targeting vector containing the *Pgk-neo* selection cassette. The insertion of this targeting vector resulted in the 4 kb deletion containing a majority of the δ-chain C-region coding sequence for the TCR.[47] Mice homozygous for the Tcrd^{tm1Mom} mutation were found to be viable and able to breed.[48] The C57BL/6J (TCR γδ$^{-/-}$ T-cell KO) strain was generated by backcrossing mice carrying the Tcrd^{tm1Mom} mutation 12 times to C57BL/6J inbred mice.[49] As a result of the knockout mutation, γδ T-cell receptor expression is deficient in all adult lymphoid and epithelial tissue. However, there

remains normal development and expression of the αβ T-cell gene and its gene product, the αβ TCR T-cell. Phenotypic markers of normal developed αβ TCR T-cell such as $CD4^+CD8^-$ and $CD4^-CD8^+$ in the periphery are maintained.[50]

Once in the Soloski Laboratory the C57BL/6J and TCR γδ T-cell knockout mice strains continued to be bred, separately. At six to eight weeks, each male offspring from each strain were segregated and used for the protocols described I this chapter.

Tissue Isolation

The uninfected tissues in the GI-tract of both the WT and KO mice, including the intestines organs and liver, were rapidly dissected from sacrificed mice and perfused in ice-cold 1X PBS 16 hours after an oral dose of sterile 1M PBS was administered to each mouse. Mesenchyme tissue and very loose connective tissue intertwined with the mesenchyme were removed to straighten and lengthen the organs of the small and large intestine. This process, performed in cold room conditions, was necessary to differentiate the tissue types of the jejunum and ileum (small intestine) and the cecum, ascending and descending colon of the (large intestine). All of these tissues were immediately snapped frozen over liquid N_2 and stored at -80°C before RNA extraction.

RNA Extraction

Total RNA was extracted using the Trizol Reagent method (Invitrogen, Carlsbad, California 92008, cat. no. 15596-026). Additional purification was performed on RNeasy columns (Qiagen, Valencia, CA 913555, cat. no. 74104). The quality of total RNA samples was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo

Alto, CA).

Microarray Analysis

RNA samples were labeled according to the chip manufacturer's recommended protocols. In brief, for Illumina, 0.5 µg of total RNA from each sample was labeled by using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX 78744-1832, cat. no. IL1791) which uses a two-step process of cDNA synthesis followed by in vitro RNA transcription. Single stranded RNA (cRNA) was generated and labeled by incorporating biotin-16-UTP. 0.75 ugs of biotin-labeled cRNA was hybridized (16 hours) to Illumina Sentrix Human HT12_v3 BeadChips (Illumina, San Diego, CA 92121-1975, cat.no. BD-103-0203). The hybridized biotinylated cRNA was detected with streptavidin-Cy3 and quantitated using Illumina's BeadStation 500GX Genetic Analysis Systems scanner.

Analytical Methods and Statistical Analysis

A single intensity (expression) value for each Illumina probe on the array was obtained using Illumina BeadStudio software with standard settings and no background correction. The expression values for all the probes for each sample were scaled to have median 256 (28) and then log (base 2) transformed before performing statistical analysis. Probes that did not have at least one sample with Illumina detection p-value < 0.01 were eliminated from further consideration. A total of 13882 probes were thus used throughout the remainder of the analysis. Genes (i.e. Illumina probes) considered to be significantly differentially expressed between two groups of samples were those satisfying the three criteria: (i) Two-sided Welch t-test p-values less than or equal to 0.01; (ii) a Benjamini-Hochberg false discovery rate (FDR) less than or equal to 0.1; and (iii) a fold change

above 2 or below -2. Functional annotation was carried out using gene lists submitted to either to the GSEA or David web-based tools, or the bioinformatics analysis tool Accumenta™ Literature Lab [http://www.acumenta.com/ acumenta/index.php] which performs a statistical analysis on PubMed abstracts to identify pathways, diseases, and compounds as they may relate to experimentally-derived gene lists. Heat maps (and the ordering by hierarchical clustering of the samples and the genes in heat maps) were based on normalization (by Z transformation) of expression values for each Illumina probe across all samples. Hierarchical clustering was performed using the Gene Cluster and TreeView software programs. The clustering algorithm was set to single linkage clustering using un- centered correlation.

Permeability Tests for Intestinal Integrity

WT uninfected mice and KO uninfected mice were orally fed with 22 mg/ml of of FITC-Dextran (Sigma) of varying molecular weights. Whole blood samples were collected four hours after feeding and centrifuged with serum separators (Becton-Dickinson). The levels of FITC-Dextran in serum were determined using a spectrofluorometer tuned to 485nm excitation and 540nm emission. FITC-Dextran sizes 10 kD, 60 kD, and 100 kD were used.

Transmission Electron Microscopy (TEM)

The mice were sacrificed with isoflurane in the chemical fume hood. Each mouse were opened from the abdomen. A polyethylene perfusion catheter was inserted into the gut just below the duodenum and secured in place by a hemostat. A collecting cannula (18 gauge, BD Sciences) was inserted into the lumen of the jejunum 10 cm further down to collect the perfusion fluid. The abdominal wall was closed. The isolated segment of

small intestine was quickly rinsed with perfusion fluid, the input catheter was attached to a perfusion pump (Minipuls II, Gilson Instruments, achieving a flow of 2 ml/15 min), and 15-min samples were collected from the output catheter. The perfusion fluid was a solution containing (in mM) 115 NaCl, 4 K2HPO4, 0.4 KH2PO4, 25 NaHCO3, 1.2 MgCl2, and 1.2 CaCl2, pH 7.4 and glucose was added at 10 mM. This solution was pre-warmed at 37°C before reach the intestinal lumen. Perfusion began with an equilibration period of 45 min. Each mouse was euthanized at the end of the perfusion, and the segment of jejunum was removed and its length of 2.0 cm measured for TEM.

In TEM a high-energy electron beam from the HT7700 120kV TEM interacts with a (~ 100-150 nm thick) specimen in order to study the morphology and composition of the jejunal tissue of the small intestine. Our specimens were fixed chemically to ensure that the specimen was preserved as close as possible to its native state. Then the specimens were dehydrated and cut into thin slices to a thickness of 250 μm. Samples with size approximately 5 cm in diameter and 10 cm in length are placed in a specimen holder that is fixed to a specimen arm, which can be raised or lowered with respect to the diamond-wafering blade. The slices can be cut with a blade with a rotation speed of about 75-100 rpm at a thickness of approximately 100-120 μm.

RESULTS

Impact of γδ T-Cell Deficiency on the mRNA profile of the GI tract

RNA was extracted from mouse intestinal epithelium isolated from the following tissues; jejunum (Je), ileum (Il), cecum (Ce), ascending and descending colon (Ac and Dc, respectively) from both WT and KO mice. Although the emphasis in this section of results and discussion is primarily focused on those WT and KO mice without

typhimurium infection, broad comparison data will at times note the experimental data for mice in the infection state. Microarray analysis was performed on six separate tissue types, two mouse phenotypes (WT and KO), and under two different conditions (with or without infection), in triplicate for a total of 72 samples. A heat map of gene expression (Figure 1.0) displays the microarray results for all data (filtered for present genes only, 13,882 genes/probes total) for the tissues of the mouse GI tract (liver samples not included) tested in this study. Remarkable patterns of differential gene expression are apparent across the range of samples, tissues, phenotypes and treatments. A distinction can be seen in both up- and down- regulated gene expression between tissues of the upper and lower GI tract (UGI and LGI, respectively) as seen in Figure 1b (IA and IB). The jejunum and ileum of the small intestine were separately grouped from the ascending and descending colon of the large intestine (with cecum as an intermediate between the two groups).

The functional annotations of these genes were done with the aid of the DAVID database from the NIH.[51,52] These tissue specific differentially expressed genes (DEGs) are also consistent with the anatomical and functional distinctions between tissues of the upper and lower GI as was predicted by the functional annotation enrichment analysis of the genes involved. The gene clusters were determined to be significant statistically if the clusters were $p \leq 0.5$ and each individual gene was given significance if $p \leq 0.5$ and the fold change was at least ± 2.0.

Gene expression which was strongly up-regulated in the LGI compared to the UGI include extracellular matrix, ($p < 1.3E-06$), and epithelium development ($p < 5.7E-05$) can potentially point to the effects of TCR γδ T-cell IELs on biological/biochemical

pathways for structural homeostasis. Additionally, gene expression which was up-regulated in the UGI versus the LGI was characterized by genes involved in intestinal absorption and the digestive system process ($p < 6.8E-05$), lipid transport and localization, ($p < 4.1E-06$), exopeptidase activity ($p < 1.3E-06$), and sugar transport ($p < 5.8E-05$), which, again, underscores the potential for TCR γδ T-cells to make an important contribution to an environmental homeostasis that is observed in healthy, functional tissue.

Impact of γδ T-Cell Deficiency on the mRNA profile of the GI tract

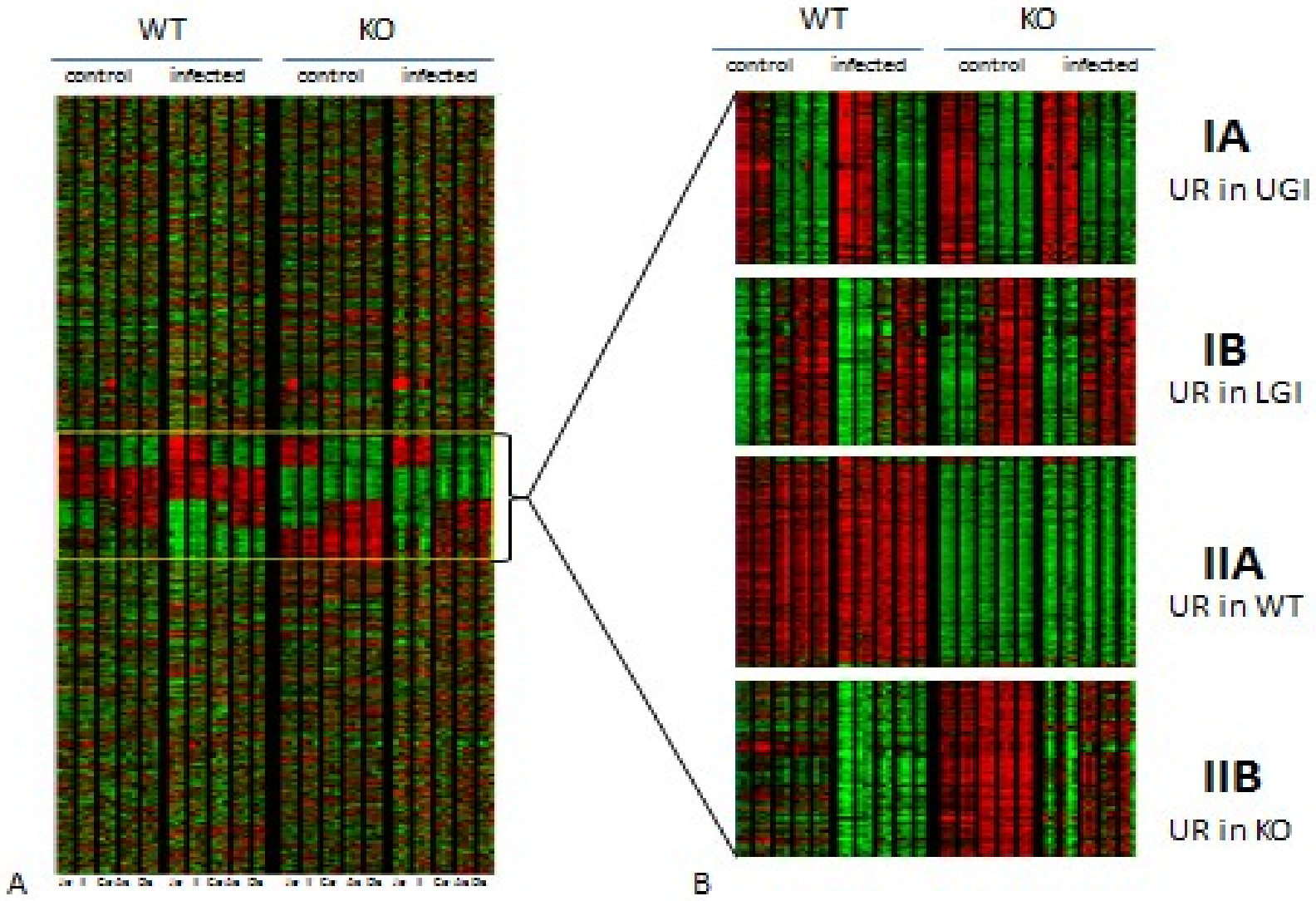
A second major pattern apparent upon examination of the data in the heat map of Figure 1.1b is the presence of large differences in gene expression between the WT and KO phenotypes which extend across all the GI tissues examined (Figure 1.1 sections 1B - IIA and IIB). It is apparent that these WT/KO DEGs represent a large shift in the overall GI transcriptome dependent upon the deletion of a single cell type the TCR γδ T-cell.

As shown in Figure 1.2, functional annotation of genes up-regulated in WT versus KO reveals an enrichment for mitochondrial genes (DAVID, $p < 9.5E-10$), cellular respiration (DAVID, $p < 2.7E-06$), and DNA repair (DAVID, $p < 2.2E-05$). The up-regulated genes from the TCR γδ KO versus the WT reveal an enrichment of genes specific for cell cycle

FIGURE 1.1: GLOBAL GENE EXPRESSION ACROSS THE GI TRACT

A. Heat map of global gene expression across all GI tract tissues. Genes/probes are row normalized across samples.

B. Zoom in of boxed area in A showing distinctive patterns of gene expression including, IA, genes up-regulated in the upper GI (UGI-jejunum and ileum) versus the lower GI (LGI-cecum, ascending and descending colon); IB, genes up-regulated in the LGI versus the UGI; IIA, genes up-regulated in WT versus KO GI tract; IIB, genes up-regulated in KO versus WT GI tract.


WT
KO
control
infected
control
infected
A
WT
KO
control
infected
control
infected
IA
UR in UGI
IB
UR in LGI
IIA
UR in WT
IIB
UR in KO
B

(DAVID, p < 6.1E-11), translation (DAVID, p < 9.9E-12), and proteolysis (DAVID, p < 3.0E-13).

Transcriptome Analysis

In Figure 1.3, differential gene expression regulated only by TCR γδ T-cells was studied by focusing more closely on the jejunum tissue of the small intestine. We began determining functional gene families with which to compare and contrast different regions of the GI tract using the same criteria used to determine up- and down-regulation of genes. These parameters include significant signal amplification with a correlating fold change of over 2.0 over or under the baseline with of significance factor of $p \leq 0.05$. Figure 1.3 shows a heat map displaying those genes expressed in the jejunum that demonstrate the greatest up-regulation in the tissue from TCR γδ deficient mice. The cluster results include super-fold increases for gene families whose protein products regulate rapid immune response; control extra-cellular matrix regulation and cell adhesion; and those that manage a family of serine/cysteine protease inhibitors (SERPINS) that affect both the rapid immune response and extra-cellular matrix modeling and remodeling.

One of the many known functions of the SERPINs is to regulate the cellular/biochemical pathways that directly or indirectly control the growth and constriction of blood vessels at the sites of injury and/or infection**.** As shown in Figure 1. 3, the Serpins with elevated expression in the TCR γδ KO are serpina1d (7.05 fold change, p=1.18E-02) and serpina1b. These Serpins are murine functional homologues to human gene SERPINA1.[53,54,55,56] SERPINA1 is a secreted serine protease inhibitor

FIGURE 1.2: FUNCTIONAL REGULATION PATTERNS ACRSOSS THE UPPER GI

Heat map of global gene expression across all GI tract tissues. Genes/probes are row normalized across samples. Zoom in showing distinctive patterns of gene expression across the GI tract for normal and TCR gamma delta T-cell deficient tissue.

WT
KO
WT
KO
mitochondrial genes (p < 9.5E-10)
cellular respiration (p < 2.7E-06)
DNA repair (p < 2.2E-05)
cell cycle (p < 6.1E-11)
translation (p < 9.9E-12)
proteolysis (p < 3.0E-13)

whose targets include elastase, which is a primary target; plasmin and thrombin for which it has a moderate infinity; and trypsin, chymotrypsin, and plasminogen activator, to which Serpina1 binds irreversibly.

Additional Serpins with elevated gene expression in the TCR γδ KO are SerpinF1 (3.94 fold change, *p*=1.37E-02), and SerpinF2 (2.20 fold change, *p*=3.12E-02). The murine genes Serpinf1 and Serpinf2 are orthologous to human genes SERPINF1 and SERPINF2.[57]

The Serpina3m gene identified as one of the up-regulated genes (Figure 1.3) has a 2.45-fold change (*p*=1.96E-0.02) in the TCR γδ T-cell KO over the regulation observed in the WT. It has been shown that the murine Serpina3m protein is regulated by the TLR-4 (Toll-like Receptor-4) signaling cascade.[58] Serpina3m is up-regulated in response to pneumonia caused by gram-negative bacteria.[59]

One of the non-Serpin family of genes that are significantly up-regulated and also spans the categories of matrix remodeling and/or the rapid immune response is the FGA gene, which has a 5.54 fold increase in expression compared to the WT counterpart (*p*=3.99E-02) as shown in Figure 1.3.

Intestinal Permeability

We have shown that the absence of TCR γδ T-cells in the gut epithelia changes the intestinal genomic profile. The majority of genes up-regulated, particularly in the jejunum of the small intestine, encode protein products that are responsible for the maintenance of ECM, cell adhesion, and clotting. A question arising from these results is

how are these differences phenotypically expressed in the proper functioning of the gut mucosa?

We hypothesized that γδ T-cells participate in the architectural and environmental maintenance of the gut epithelial layer. These functions ensure that the tissues involved in creating crucial barriers and basic functionality for the gut can be kept intact. The epithelial barriers function for the active and selective diffusion of minerals and metabolites across cell matrices Because of this intestinal function, each tissue in the normal GI tract has a polarity and selectively when transporting molecules across the epithelial barrier. To hypothesize this, we tested the permeability of the epithelial layer was tested using FITC-conjugated dextran substrates of varying molecular weights in Figure 1.4. The sizes of the molecules to be tested were 4 kD, 10kD, 60kD, and 100kD.

Displayed in Figure 1.4 are the levels of FITC found in the serum of the wild-type and TCR γδ deficient mice after oral administration of FITC-Dextran of varying molecular weights. The intestine of the TCR γδ-deficient mice display increased permeability to the lower (4kD and 10 kD) but not the higher molecular weight dextrans. This implies that the absence of TCR γδ cells in the gut mucosa does alter permeability. However, the changes are such that only small molecule transport is affected. Since tight junctions play a key role in regulating small molecule movements through the gut epithelium, the results suggest that there may be changes in tight junction density or structure.

TEM of Gut Epithelial Layer

The permeability studies above revealed a role for TCR γδ T-cells in mounting a barrier function in the gut epithelial. To further examine this we studied the gut epithelial

structure using TEM. Figure 1.5a shows a normal wildtype intestinal epithelial tissue from the jejunum. There are distinct patterns of columnar formation between adjacent intestinal cells. Cellular boundaries are observed that demarcate the apical and baso-lateral sides of the intestinal barrier.

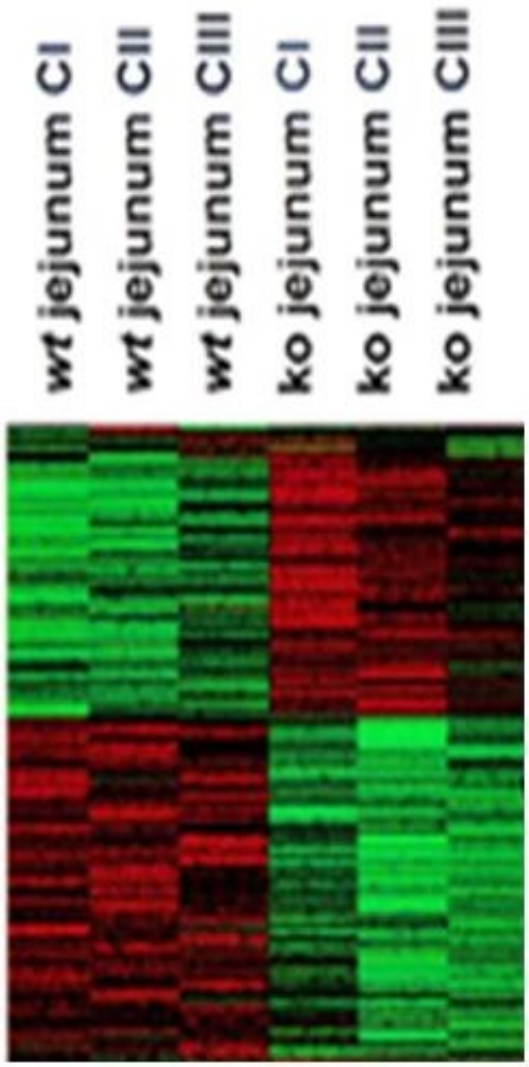
In contrast, Figure 1.5b shows that the tissue lacking TCR γδ T-cells have a more poorly defined columnar appearance. The intestinal tissue as seen under electron microscopy shows greatly narrowed intestinal epithelial cells with an unusual channeling between the apical and lateral borders that are distinct from normal epithelial tissue. Overall, the architecture and morphology of the actual intestine of the KO before infection is that of a distinctly different morphological tissue than its WT counterpart.

DISCUSSION

Deletion of γδ T-cells from the gut GI tract has large transcriptomic and functional consequence. These programmatic shifts are large and fundamental and as distinctive in their way as the changes in gene expression which distinguish upper and lower GI (Fig 1.1). The transcriptional profile of the upper GI was characterized by gene groups involved in lipid transport and metabolism (glycerol and cholesterol metabolism, triglyceride metabolic process), digestive system process, and endoplasmic reticulum all consistent with the role of these tissues in digestion and absorption. The transcriptional profile of the lower GI was distinguished by gene groups involved in extracellular matrix, contractile fibers, and epithelium development attributable to the maintenance and

FIGURE 1.3: FUNCTIONAL CLUSTERING OF UP-REGULATED GENES IN JEJUNAL TISSUES OF WT AND TCR γδ T-CELL DEFICIENT IN MICE

Heatmap taken from tissues of the jejunum in the small intestine of both the WT and TCR γδ deficient mice. Differentially upregulated gene clusters in the γδ deficient mice include the Sepins, genes of the ECM/Adhesion, and those of rapid immune response.



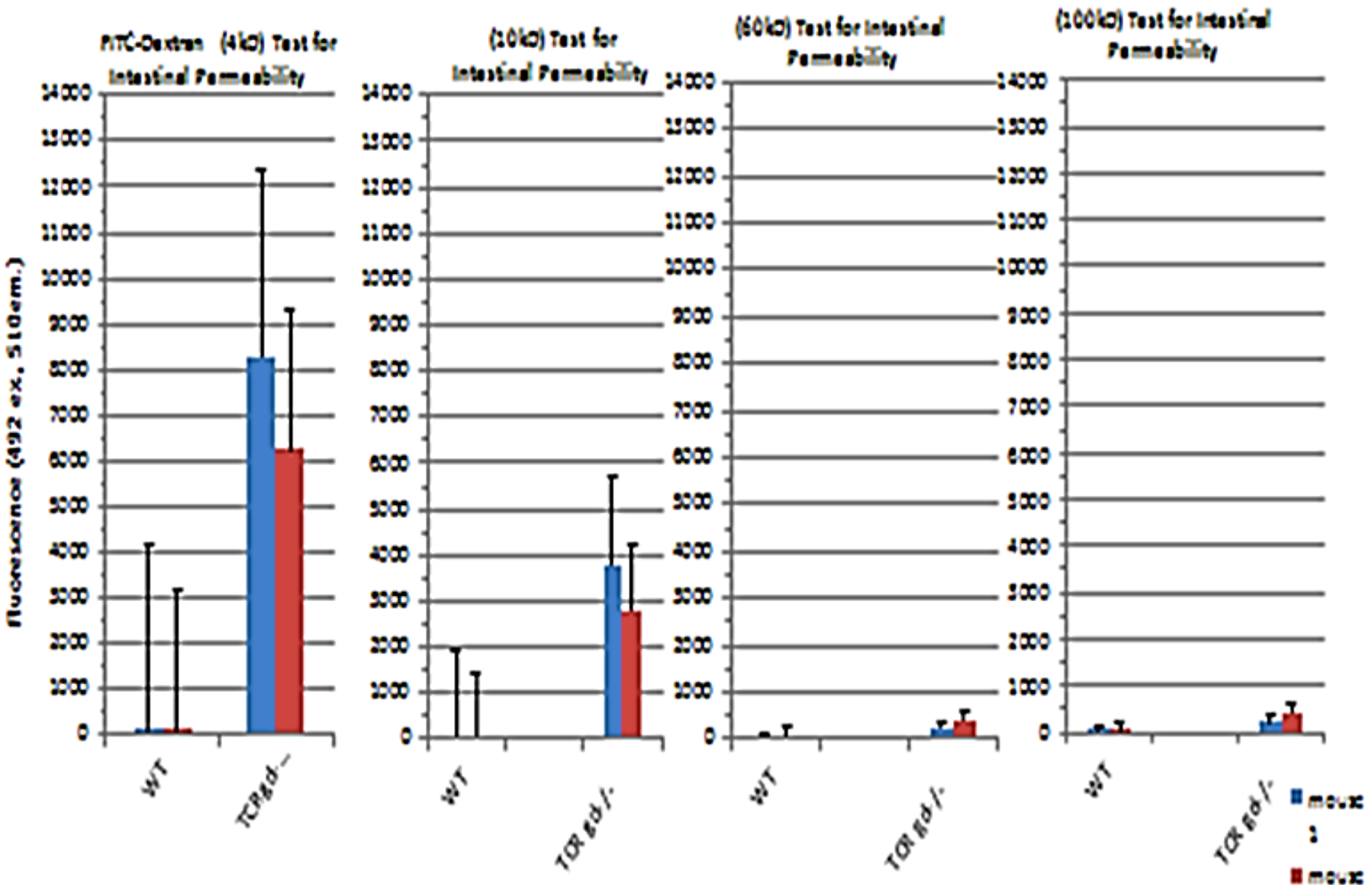
Serpin Family	Fold Increase	*p*-value
Serpina1d	7.05	1.18E-02
Serpina1b	6.71	1.26E-02
Serpina1e	5.77	4.43E-02
Serpind1	2.60	1.59E-02
Serpina3m	2.45	1.96E-02
Serpinf1	3.94	1.37E-02
Serpinf2	2.20	3.12E-02

ECM/Adhesion	Fold Increase	*p*-value
Serpina1d	7.05	1.18E-02
Serpina1e	5.77	4.43E-02
Fga	5.54	3.99E-02
Apoh	5.00	3.04E-02
Serpinf1	3.94	1.37E-02
vtn	3.48	2.79E-02
Lama3	3.43	2.76E-05

Rapid Immune Response	Fold Increase	*p*-value
Fga	5.54	3.99E-02
Serpina3m	2.45	1.96E-02
Lama3	3.43	2.76E-05
Apoh	5.00	3.04E-02
Gasta3	5.19	1.13E-02
vtn	3.48	2.79E-02
Lyzs	3.01	3.38E-07

FIGURE 1.4: EFFECTS OF INCREASING MOLECULAR WEIGHT FITC-DEXTRAN SIZE ON PERMEABILITY

Age-matched wildtype and TCR gd deficient mice were orally gavaged with 2.2mg/ml of varying molecular weights of FITC-Dextran Serum was isolated 16 hours later and the FITC-Dextran signal was detected spectrophotometrically at 492 nm. The permeability of the epithelial barrier is shown FITC-Dextran using a) 4KD, b) 10 KD, c) 60 KD, and, d) 100KD.


FITC-Dextran (4kD) Test for Intestinal Permeability
(10kD) Test for Intestinal Permeability
(60kD) Test for Intestinal Permeability
(100kD) Test for Intestinal Permeability
Fluorescence (492 ex., 510em.)
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
WT
TCRgd-/-
mouse 1
mouse 2

renewal of the large intestinal glands in colon. The clear separation of upper and lower GI WT and TCR γδ T-cell deficient mice would be possible. The transcriptional evidence of large programmatic shifts favoring energy metabolism in WT tissues and accelerating cell division in KO across multiple tissues suggests that a profound shift in the homeostatic balance of the GI tract has taken place as a result of the deletion of TCR γδ cells and by inference as well suggests a profound regulatory role for this specialized immune cell in normal GI function.

The results presented here demonstrate that, the transcriptional program has been profoundly and specifically altered across the multiple tissues of the GI tract in the absence of γδ T-cells. The removal of the γδ T-cell from the gut environment leaves an unmistakable biological footprint which impacts maintenance/modeling of the GI tract environment and potentially effects pathogenic processes such as response to infection. The latter point will be the subject of the next chapter. Significant adverse effects of the loss of γδ T-cell to normal GI function include a change in gut epithelial permeability that we demonstrate in Figure 1.4. This result implies that a significant degree of disruption has occurred in the absence of TCR γδ T-cells.

Transcriptome Analysis

Our studies have shown that a number of members of the Serpin gene family are up-regulation and by the sheer number of genes up-regulated in the GI tract of TCR γδ -deficient mice. These findings show this family of genes must have significant roles in the regulation of metabolic processes, rapid immune responses, and ECM modeling that ensures gut epithelial homeostasis. The up-regulation of these genes in the absence of

FIGURE 1.5: ELECTRON DENSITY OF THE EPITHELIAL LAYER OF THE JEJUNUM OF THE SMALL INTESTINE

Transmission Electron image of a cross-section of a murine jejunum epithelial cell. A) Image shows normal tissue from the apical border to basal side. There is the distinctive columnar appearance of healthy intestinal cells. B) Image shows Uninfected KO intestinal tissue that has a less columnar appearance and poorly formed boundaries of the apical and baso-lateral sides.

B
A

TCR γδ T-cells in the gut holds a possible clue to the broader implications of the T-cell subset in this environmental homeostasis needed for proper functioning of the gut epithelia. The growth and constriction of blood vessels and the protein cascades that control them are the lead factors of wound clotting, which is a major regulatory component of balance and maintenance of the epithelial layer.

Several genes are identified in Figure 1.3 are classified as Rapid Immune Response genes that are part of defense systems or signaling cascades that are involved in the host response to injury or infection. Some of these signaling cascades help to recruit and traffic innate immune cells to the site of infection or injury and will model and remodel the epithelia to achieve these ends. Others have a role in immune surveillance.

Three murine Serpin genes show significantly higher signals in the GI tract in the absence of TCR γδ T-cells. The three genes are serpina1d, serpina1b, and serpina1e. These Serpins are remarkable because they are murine functional homologues to human gene SERPINA1.[60,61,62,63] The protein encoded by this gene in humans is a secreted serine protease inhibitor whose targets include elastase, plasmin, thrombin, trypsin, chymotrypsin, and plasminogen activator, to which Serpina1 binds irreversibly.[64,65,66] All of these protein targets are found prominently in the clotting pathways since they negatively and positively regulate the body's response to tissue injury or infection (or injury due to infection). The overrepresentation of signaling in murine gut epithelia of mice without TCR γδ T-cells suggests that in the absence γδ T-cells there is a constant matrix remodeling and/or regeneration needed in the epithelial barrier.is needed without the presence of the T-cell subset.

A function of the γδ T-cells in the gut is as a regulator of the rapid immune response and immune surveillance. [67,68] The Serpin gene Serpin a3m is up-regulated in the TCR γδ T-cell knockout. The Serpina3m protein is regulated by another part of the host innate immune defense system, the TLR-4 signaling cascade.[69] The TLRs are pattern-recognition receptors that recognize pathogen-specific structures. The ligand for the TLR-4 receptor is lipopolysaccharide (LPS, endotoxin) which is on most Gram-negative bacteria such as *S. typhimurium*. Activation of TLRs results in the induction of a potent pro-inflammatory response, including the release of pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, TNFα and IL-8.

The TLR-4 signaling angle is important as it relates to the up-regulation of the gene Serpina3m in the gut epithelia of the mice lacking the TCR γδ T-cells. A very important point to emphasize is that these results suggest that in the gut, the TCR γδ T-cells may serve as surveillance against impeding pathogen invasion.

A subset of γδ T cells that produce IL17, have recently been shown to have tumor-promoting functions including immuno-surveillance and modulation in colorectal cancers and breast cancer metastasis models. [70,71] Interestingly, the immune modulation properties of the IL 17 TCR γδ T cells were revealed in the disruption of the epithelial barriers that were breached by the invasion of microbial pathogens. This disruption of the epithelial barrier correlated with inflammatory dendritic cell activation which secretes IL-23, thus promoting γδT17 polarization. [72] Activated γδT17 cells also secreted other cytokines including IL-8, tumor necrosis factor alpha (TNF-α), and GM-CSF, which might chemoattract polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) in the tumor and sustain their immunosuppressive activity.[73]

Without the population of γδ T-cells in the mix of iIEL's in the gut, the KO intestines are forced to compensate with up-regulating genes whose protein products are partly responsible for immune-surveillance in TCR γδ T-cell deficient mice that are otherwise uncompromised by infection or injury.

Additional Serpins with elevated gene expression in the TCR γδ KO are SerpinF1 and SerpinF2. The murine genes Serpinf1 and Serpinf2 are orthologous to human genes SERPINF1 and SERPINF2.[74] The protein product of SERPINF1, also known as pigment epithelium-derived factor (PEDF), is a secreted protein that is a non-inhibitory neurotrophic factor, and an angiogenesis and tumorigenesis regulator.[75, 76, 77, 78] The protein SERPINF2, also known as alpha–2–plasmin inhibitor, is responsible for inactivating plasmin, an enzyme that has a crucial role in fibrinolysis, which is the process of preventing blood clots.[79] This infers that γδ T-cells have a potent role in modeling and remodeling of the intestinal environment that the intestine must compensate for when these T-cells are absent.

Another gene that had a high intensity signal was the gene FGA encoding the fibrinogen alpha chain. The importance of this protein product is that its function spans the categories of ECM/adhesion and rapid immune response. The protein product encoded by this gene is the alpha component of fibrinogen, a glycoprotein that is carried in the blood and is comprised of three pairs of non-identical but homologous polypeptide chains.[80] To begin the process of repair after vascular injury, fibrinogen is cleaved by thrombin to form fibrin which is the most abundant and critical component for formation of blood clots.[81,82,83] The role this protein plays in the immune response after injury is vital because the process by which immune cells first encounter damaged cells due either

to injury or the infections that cause injury is first started by that formation of clots, which carry immune cells to the site of interest through the circulatory system. The detailed steps that show how FGA cleaves fibrinogen and fibrin explain the intricate regulation that occurs when immune cells must travel to the site of injury by way of clot formation. The implication of FGA being a major component of the jejunal tissue of the small intestine in the absence of TCR γδ T-cells is that γδ T-cells have a major role in regulating and modeling the intestinal environment for a tissue specific rapid immune response.

Permeability of Epithelial Layer

We have shown that the absence of TCR γδ T-cells in the gut epithelia changes its genetic profile. The majority of genes up-regulated, particularly in the jejunum of the small intestine encode protein products that are responsible for the maintenance of ECM, cell adhesion, and clotting. The question from these results is if there are phenotypic expressions of these genetic changes the proper functioning of the epithelial layer?

One of our working theories for γδ T-cell function is that this particular subset of T-cells are responsible for the architectural and environmental maintenance of the gut epithelial layer. These functions ensure that the tissues involved in creating crucial barriers and basic functionality for the gut can be kept intact. The epithelial barriers function for the active, selective diffusion of minerals and metabolites across cell matrices. Because of this function each tissue across the GI tract has a polarity and selectively when transporting metabolites across the epithelial barrier. We tested permeability of the epithelial barrier using FITC-conjugated dextran polymers.

Our results indicate that in the absence of TCR γδ T-cells, the gut epithelium displays increased permeability to lower weight dextran polymers. In contrast, the intestinal membranes of each set of mice, regardless of the presence of TCR γδ T-cells, restricted the diffusion of the dextran polymers of a higher molecular weight.

TEM of Gut Epithelial Layer

Since the membrane permeability tests showed differences in ion migration across the epithelial layer in mice that TCR γδ T-cells, our working hypothesis is that an alteration in the formation of tight junctions may be the consequence of missing γδ TCR regulation in the gut epithelia. In wild-type mice, there are well defined, columnar intestinal epithelial cells of the jejunum with villa protruding from the apical side along the brush border. The tissue from wild-type mice has good junctional definition (tight junctions, adherens junctions, desmosomes and gap junctions) and well-developed demarcation of the apical and baso-lateral sides of the intestinal tissue.

This tissue architecture type is typical of intestinal epithelia where there is a regulated exchange of nutrients, macromolecules, and gases coming from the intestinal lumen to the bloodstream. Just proximal to brush border of the apical (or lumen) side of the epithelia, molecules first encounter tight junctions. Through the process of receptor-mediated endocytosis, the tight junctions will selectively allow molecules to be actively transported from the apical villus to the epithelial layer of the tissue. Once the molecule transverses the epithelial layer, exocytosis delivers nutrients and molecules to the bloodstream. The well-developed architecture of the epithelial layer underscores the regulated and normal functioning of the WT gut epithelia.

However there are distinct changes in the appearance in morphology and architecture of the intestinal tissue lacking the TCR γδ T-cells. There is a broad lack of columnar definition that forms the basic boundary for the intestinal cell. The tissue lacking γδ T-cells also show a unique and unusual excess channeling with no pattern found in the architecture of a well-functioning, normal WT epithelial tissue. These morphological observations are consistent with some of the subtle increase in the permeability or "leakiness" observed in the tissues lacking TCR γδ T-cells. The architectural changes seen underscore the lack of regulation of some molecules across the barrier as well as help tether the intestinal cell to the matrix of the tissue. More broadly, these results indicate that this particular iIEL population may directly affect the maintenance and regulation of gut epithelial function by controlling the basic development of morphology and architecture of intestinal tissue.

CONCLUSION

Deletion of γδ T-cells from the environment of the gut has large phenotypic consequences for gene transcription, structural architecture and maintenance, and functionality. Functional annotation analysis of the KO genes reveals a very significant enrichment for genes of the acute phase response (DAVID, $p < 7.4E-24$). This response is both specific to the KO mouse jejunum. It is apparent that the deletion of TCR γδ T-cells from the mouse jejunum not only prevents a normal response pattern in these mice, but renders a genetic footprint that is distinctive from the genetic markers and function found in the normal jejunal tissue of the small intestine. Therefore the lack of γδ T-cells renders the small intestine, particularly the jejunum, a different tissue in biological and genetic expression.

While examining the broad gene expression patterns, we looked at highly up-regulated individual genes for clues as to how the presence of TCR γδ T-cells help to maintain function and structure of the gut epithelial layer. Among the genes that were up-regulated were the Saa genes which are the first responders to injury and infection and Serpin genes such as Serpina2m and Serpinf2, which have important roles in regulation the vascularization and clotting needed for remodeling the epithelium to respond to infection and injury. The positive regulation of these gene families in TCR γδ T-cell deficient jejunum may indicate that the otherwise normal tissue is under a constant state of duress. To compensate for the loss of TCR γδ T-cells there is an up-regulation of genes that will protect against biological insult and to perform modeling/remodeling.

CHAPTER 2

The Impact of TCR γδ T-cell Deficiency on the Response of the GI Tract to *Salmonella enterica typhimurium* Infection: Whole Tissue Genomic and Phenotypic Analysis

INTRODUCTION

The previous set of studies in Chapter 1 indicate γδ TCR T-cells have an integral role in the maintenance of the integrity of the epithelium barrier.[84,85,86] In this chapter we further explore this by examining the impact of removing TCR γδ T-cells in the gut epithelium during the initial host response to infection. This approach has shed light on the role of TCR γδ T-cells in the structural, architectural, environmental homeostasis; and functioning of the intestinal gut mucosa.

The Pathogen-Salmonella

Salmonella enterica typhimurium is the pathogen of choice for our laboratory to examine the function of TCR γδ T-cells in the GI epithelial barrier. The pathogenesis of infectious diseases caused by the bacterial serovar *Salmonella enterica* is based on a significant number of fimbria and non-fimbria adhesin molecules that mediate biofilm formation and attachment to host cells. Secreted proteins are also involved in host cell invasion and intracellular proliferation, two hallmarks of *Salmonella* pathogenesis. *Salmonella enterica* has six subspecies, and each subspecies has associated serovars that differ by antigenic specificity. There are over 2500 serovars for *S. enterica*. Most of the vertebrate pathogenic Salmonella serovars belong to serogroups that include *Salmonella Typhi, Salmonella Enteritidis, Salmonella Paratyphi, Salmonella Typhimurium*, and *Salmonella Choleraesuis*. The serovar Typhi is pathogenic in humans while *Typhimurium* mimics the disease progression in murine species.[87]

METHODS

Tissue Isolation

The tissues in the GI-tract of both the WT and KO mice, including the intestines organs and liver, were rapidly dissected from sacrificed mice and perfused in ice-cold 1X PBS 16 hours after an oral dose of sterile 1ml PBS was administered to each mouse. Mesenchyme tissue and very loose connective tissue intertwined with the mesenchyme were removed to straighten and lengthen the organs of the small and large intestine. This process, performed in cold room conditions, was necessary to differentiate the tissue types of the jejunum and ileum (small intestine) and the cecum, ascending and descending colon of the (large intestine). All of these tissues were immediately snapped frozen over liquid N_2 and stored at -80°C before RNA extraction.

In vivo infection with S. typhimurium for Microarray

Eight- to 10-wk-old gender-matched mice were either mock infected with sterile PBS or infected orally with 5×10^5 wild-type or 5×10^8 aroA− Salmonella in 0.1 ml of PBS, using a standard gastric intubation needle. At various times after infection, small intestine, mesenteric lymph nodes (MLN), PP, liver, and spleen were isolated from infected and control animals. The number of bacteria present in each mouse strain was determined by preparing single-cell suspensions from the mesenteric lymph nodes (MLN), Peyer Patches (PP), liver, and spleen using a Stomacher (Seward, London, U.K.), and making serial dilutions in PBS of the suspensions obtained. Aliquots of each serial dilution were grown on TS agar plates.

RNA Extraction and Microarray Analysis

Total RNA was extracted using the Trizol Reagent method (Invitrogen, Carlsbad, California 92008, cat. no. 15596-026). Additional purification was performed on RNeasy

columns (Qiagen, Valencia, CA 913555, cat. no. 74104). The quality of total RNA samples was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Further details are located in Chapter 1.

Quantitative RT-PCR (QRT-PCR) Analysis

cDNA was obtained from total RNA using the cDNA Archive Kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Probes and primers were designed and synthesized by Applied Biosystems. All PCR amplifications were carried out in triplicate on an ABI Prism® 7300 Sequence Detection System, using a fluorogenic 5' nuclease assay (TaqMan® probes). Relative gene expressions were calculated by using the $2^{-\Delta\Delta Ct}$ method as described in. The ΔCt value of each sample was calculated using 3 endogenous control genes (GAPDH, ACTB, and PGK1).

Analytical Methods and Statistical Analysis

A single intensity (expression) value for each Illumina probe on the array was obtained using Illumina BeadStudio software with standard settings and no background correction. Further details are located in Chapter 1.

Permeability Tests for Intestinal Integrity

Six- to eight-week-old gender-matched mice were either mock infected with sterile PBS or infected orally with 5×10^8 CFU of aroA$^-$ *Salmonella typhmurium* in 0.1 ml of PBS, using a standard oral gavage. Whole blood samples were collected four hours after feeding and centrifuged with serum separators (Becton-Dickinson). The number of bacteria present in each mouse strain was determined by preparing single cell suspensions

in serial dilutions in PBS. See Chapter 1: "*Permeability Tests for Intestinal Integrity"* for more details.

Transmission Electron Microscopy (TEM)

Six- to eight-week-old gender-matched mice were either mock infected with sterile PBS or infected orally with 5×10^8 CFU of aroA$^-$ *Salmonella typhmurium* in 0.1 ml of PBS, using a standard oral gavage. Whole blood samples were collected four hours after feeding and centrifuged with serum separators (BD). The number of bacteria present in each mouse strain was determined by preparing single-cell suspensions in serial dilutions in PBS. Aliquots of each serial dilution were grown on TS agar plates to confirm infection. See Chapter 1 "*Transmission Electron Microscopy (TEM)"* for more details.

RESULTS

Transcriptional Analysis: Response to Infection

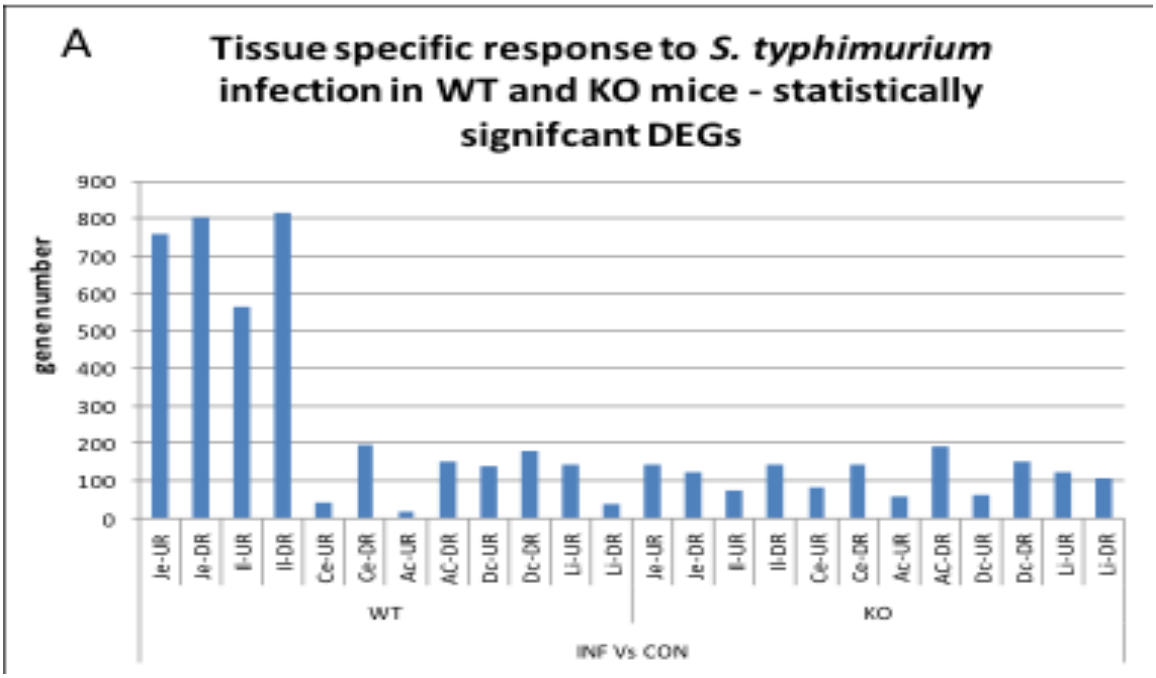
We next examined the effect on transcription in the gut of both WT and TCR γδ T-cell deficient mice in response to infection by *S. typhimurium.* We noted a large difference in response patterns between the two phenotypes across all the tissues of the GI tract, but most particularly in the jejunum and ileum, the known active site of infection for this pathogen. Figure 2.1A illustrates a dramatic shift upwards in the numbers of statistically significant DEGs in WT jejunum and ileum, for both up- and down-regulated genes, in response to *Salmonella* infection as compared to either other WT tissues (cecum, ascending and descending colon, and liver). The numbers of WT jejunum and ileum DEGs are also greater than for TCR γδ T-cell deficient jejunum and ileum tissues and the remainder of the TCR γδ T-cell deficient GI tract and liver tissues.

The specific DEGs between KO and WT was small for genes up-regulated by infection in both the jejunum and ileum (10.5% and 16.2%, respectively) but surprisingly larger both the jejunum and ileum (10.5% and 16.2%, respectively) but surprisingly larger for down-regulated genes for the same tissues (for jejunum- 66.9%, and ileum- 39.1%) suggesting that the dysregulation correlating to the deletion of TCR γδ cells primarily affects the positive transcriptional response to infection in this model system.

Figure 2.1B displays a heat map of the average Pearson correlation value for all pair wise comparisons of tissue specific responses to *Salmonella* infection (the gene expression differences for the entire dataset were used in this analysis) for both WT and TCR γδ T-cell deficient mice. Interestingly, while both the amplitude and the specificity of response to *S. typhimurium* infection are substantially altered between the WT and TCR γδ T-cell deficient mice at the level of DEGs (which measures only statistically significant changes in gene expression), the overall response at the full dataset level is still sufficiently concordant as to suggest that these response patterns still remain somewhat related across phenotypes. Gene regulation patterns (Fig. 2.1B) are seen to be highly correlated in the jejunum and ileum for both WT and separately for KO animals in response to infection, once again emphasizing both the close functional as well as physical relationship shared between these two tissues, a relationship that survives large systemic alterations in overall biological response patterns. Broad functional group characterizations of the transcriptional response to *Salmonella typhimurium* infection in WT mouse jejunum appears to have the largest increases in the metabolic function

FIGURE 2.1: GENE EXPRESSION ACROSS GI TRACT AND LIVER TISSUES IN RESPONSE TO *SALMONELLA TYPHIMURIUM* INFECTION

Comparison of statistically significant changes in gene expression across all mouse GI and liver tissues in response to Salmonella infection. **A.** Number of genes significantly regulated showing, in particular, large numbers of both up- and down-regulated genes in the UGI (jejunum and ileum) of WT but not in KO mice. **B.** Heat map of correlation values for all calculated (for the full dataset) changes in gene expression compared with one another. WT and KO are correlated despite not sharing many statistically significant DEGs. DEGs for liver are distinct from all other GI tissues.


A
Tissue specific response to S. typhimurium infection in WT and KO mice - statistically signifcant DEGs
gene number
900
800
700
600
500
400
300
200
100
0
Je-UR
Je-DR
Il-UR
Il-DR
Ce-UR
Ce-DR
Ac-UR
AC-DR
Dc-UR
Dc-DR
Li-UR
Li-DR
Je-UR
Je-DR
Il-UR
Il-DR
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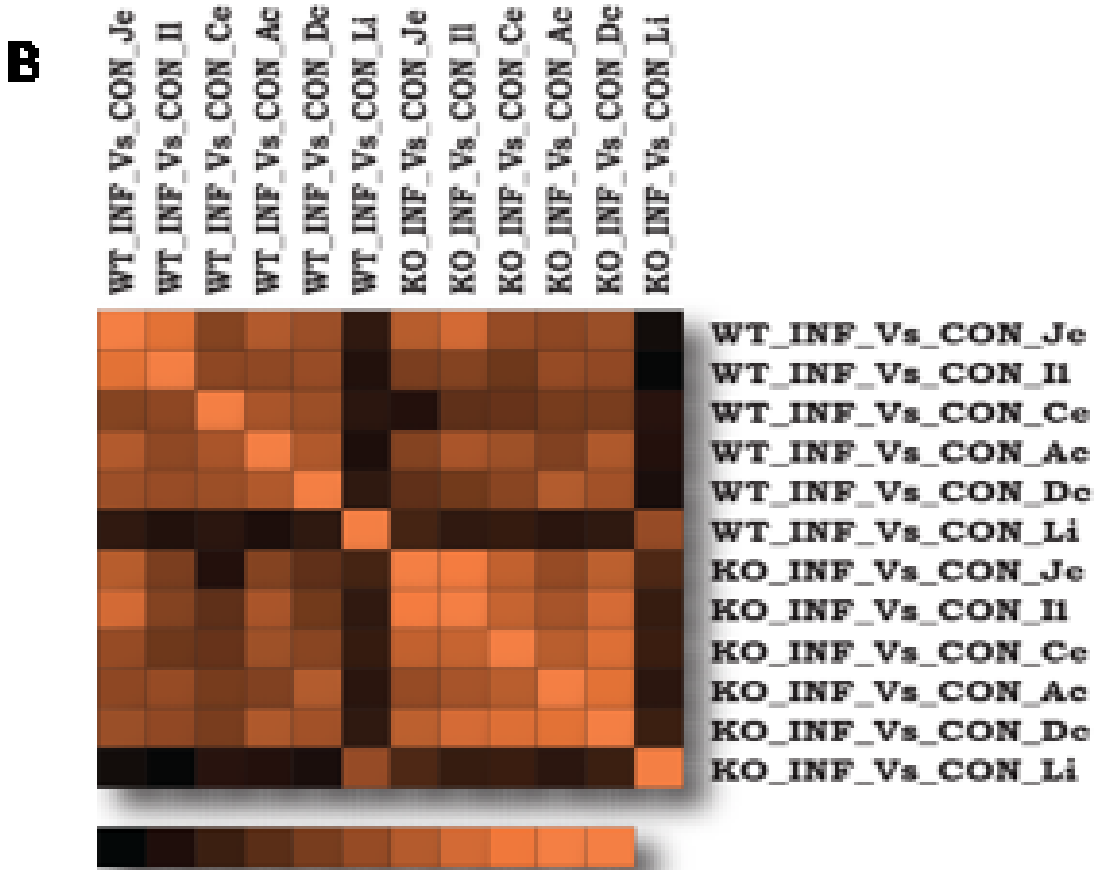
Ce-DR
Ac-UR
AC-DR
Dc-UR
Dc-DR
Li-UR
Li-DR
WT
KO
INF Vs CON


B
WT_INF_Vs_CON_Je
WT_INF_Vs_CON_Il
WT_INF_Vs_CON_Ce
WT_INF_Vs_CON_Ac
WT_INF_Vs_CON_Dc
WT_INF_Vs_CON_Li
KO_INF_Vs_CON_Je
KO_INF_Vs_CON_Il
KO_INF_Vs_CON_Ce
KO_INF_Vs_CON_Ac
KO_INF_Vs_CON_Dc
KO_INF_Vs_CON_Li

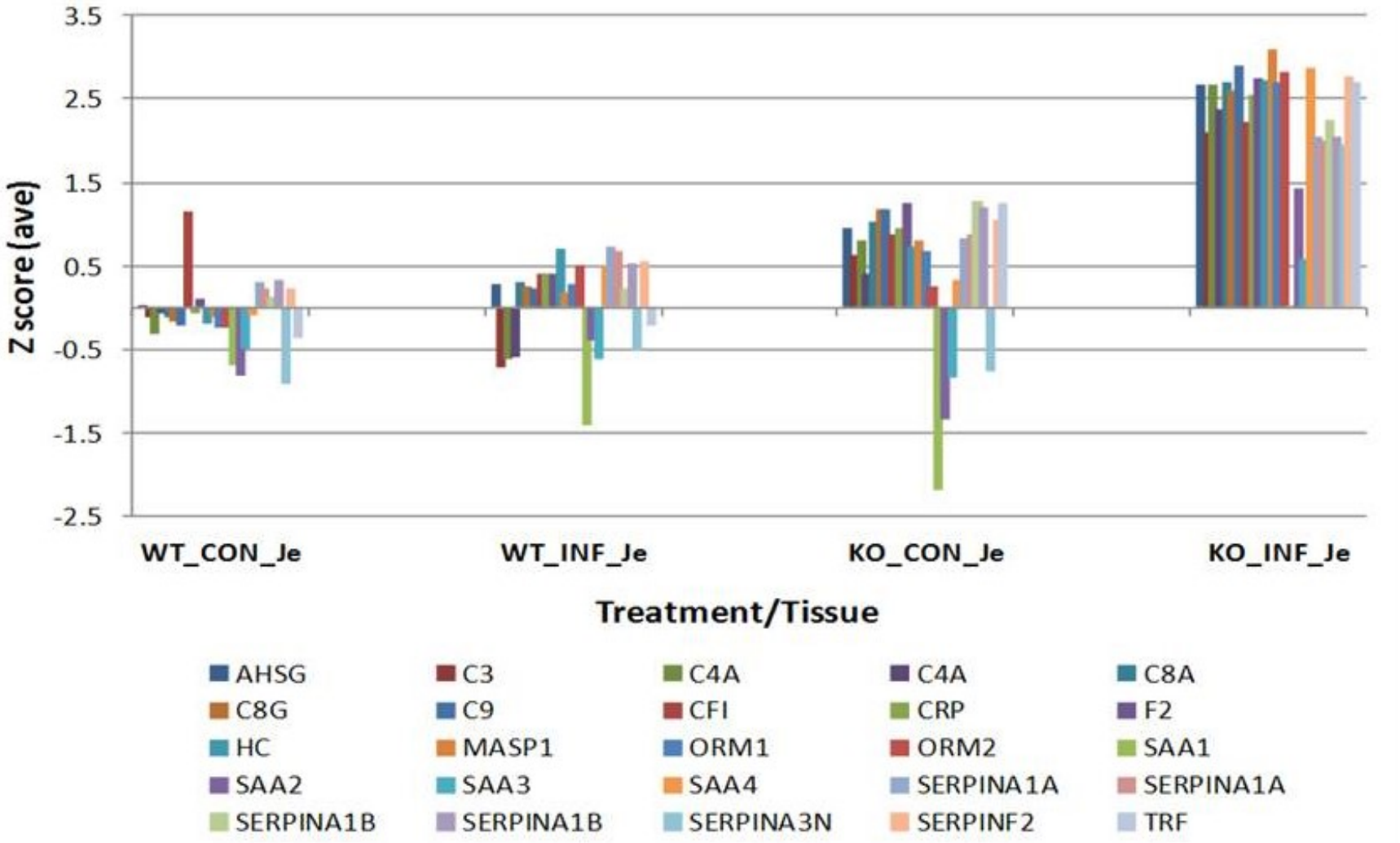
consistent with the role this tissue plays in intestinal absorption rather than the direct involvement of immune response genes at this point the infection cycle.

Affected pathways include 19 genes involved in lipid metabolism (DAVID, $p < 7.2E-08$), 84 genes annotated for transport (DAVID, $p < 6.8E-06$), 22 genes active in the monosaccharide metabolic process (DAVID, $p < 6.7E-07$) and 6 genes directly related to the digestive system (DAVID, $p < 3.6E-06$). Genes up-regulated by infection in the WT mouse jejunum actually identify that specific tissue type using an alternative text-based gene/term association tool (Accumenta, $p < 3.4E-04$). In contrast to WT, genes up-regulated in the TCR γδ T-cell deficient jejunum display none of the metabolic responses seen in the WT mice in response to infectious challenge. The infected jejunum of the γδ T-cell deficient strain show a close association with multiple disease states that denotes a type systemic organ/tissue failure post-infection including appendicitis (Accumenta, $p < 5.1E-04$), acute kidney injury (Accumenta, $p < 5.0E-04$) and liver disease (Accumenta, $p < 1.0E-03$).

Functional annotation analysis of the γδ T-cell KO genes reveals a very significant enrichment for genes of the acute phase response (DAVID, $p < 7.4E-24$). This response is both specific to the KO mouse jejunum and, as can be seen in Figure 2.2, are already elevated at baseline and become even more amplified following infection. It is apparent that the deletion of γδ T-cells from the mouse jejunum not only prevents a normal response pattern in these mice but also is associated with an extreme and pervasive pathological response, one which may contribute directly to the evident morbidity of this KO phenotype following infection.

FIGURE 2.2: THE AMPLITUDE OF THE RESPONSE OF ACUTE PHASE GENES TO *SALMONELLA TYPHIMURIUM* INFECTION

Comparison of the amplitude of the response of acute phase genes to *Salmonella* infection starting from uninfected baseline in both WT and KO jejunum. Gene expression is expressed as the average (of 3 biological replicates) of row normalized data for each gene/probe. Baseline expression of these selected genes is higher in KO than WT and becomes greatly amplified in KO together following infection.


3.5
2.5
1.5
0.5
-0.5
-1.5
-2.5
Z score (ave)
WT_CON_Je
WT_INF_Je
KO_CON_Je
KO_INF_Je
Treatment/Tissue
AHSG
C3
C4A
C4A
C8A
C8G
C9
CFI
CRP
F2
HC
MASP1
ORM1
ORM2
SAA1
SAA2
SAA3
SAA4
SERPINA1A
SERPINA1A
SERPINA1B
SERPINA1B
SERPINA3N
SERPINF2
TRF

Specifically, Figure 2.2 shows that Serum Amyloid A (Saa1/Saa2) is part of another gene family of interest in the rapid immune response super-upregulated in the TCR γδ T-cell deficient jejunum in response to infection. The elevation of inflammatory response genes in the KO mouse is most striking in the jejunum.

Figure 2.2 also shows the genes of particular interest upregulated prominently in the jejunum of KO intestines in the acute inflammatory response include a member of the Serpin family, Serpina1. This member of the Serpin gene family is part of gene isoform shown to be significantly up-regulated in the jejunum of uninfected KO intestines. The gene up-regulation measured 5-fold or above in the TCR γδ KO.

In Figure 2.2, the functional analysis of the gene profile of TCR γδ T-cell deficient mouse strain also reveals a very significant super-enrichment for genes of the acute phase response. This response is both specific to the KO mouse jejunum and is already elevated at baseline and becomes even more amplified following *S. typhimurium* infection. It is apparent that the deletion of TCR γδ T-cells from the mouse jejunum prevents a normal jejunum tissue response pattern to infection.

This is displayed in Figure 2.3 where the elevation of gene expressions is reflected across the GI tract but is most striking in the jejunum. Additional KO specific pathway involvement includes the complement and coagulation cascades (DAVID, $p <$ 9.9-23), cytochrome P450 (DAVID, $p <$ 1.6-09), and HDL (DAVID, $p <$ 1.4-18) pathways.

Figure 2.4 highlights the genetic profile displayed in Figure 2.3, with the KO jejunum showing the super-upregulation in response to infection of genes that are for

acute or rapid immune response pathways. Gene patterns for rapid immune response in the KO also have super-upregulation of gene families involved in the ECM/adhesion and clotting cascades. Among the genes that highlight this fact is the increased positive regulation observed in the genes already up-regulated in the uninfected KO jejunum. In Figure 2.1, the Serpins that have roles in both the inflammatory/rapid immune response and the underlying structural remodeling of the gut epithelia is SerpinF1.

The gene patterns seen for rapid immune response in the KO intestines also have greater implications for the regulation of genes involved more closely with the ECM and its structural maintenance. Figure 2.4 also shows the emergence of an important family of genes whose protein products are involved directly in the structure, maintenance, and functioning of the extracellular matrix in intestinal tissue. While not super-upregulated in the KO as a response to infection, the Claudins, Cldn1 and Cldn2 are still significantly elevated. The protein encoded by Cldn1 is an important integral, transmembrane protein and a component of tight junction strands.[88] Claudins are important to the function of intestinal epithelia because the help form tight junctions, and the tight junctions, in turn, are directly involved in regulating the diffusion of molecules across the epithelial barrier. Through the formation of tight junctions, claudins are directly involved in creating a primary barrier to the paracellular diffusion of solutes and water across the epithelia.[89,90] Recent studies have disclosed that claudins are the major determinant of the barrier function of tight junctions.[91]

Figure 2.5 shows RT-PCR data for validating the microarray results for the pro-inflammatory and rapid immune response genes Saa1/Saa2, whose proteins help in immune surveillance, and the Serpina1m and Serpinf1 genes, whose proteins have roles

in modeling and remodeling the gut epithelia. Over-expression of the acute inflammatory genes specifically in the jejunum of KO but not WT mice in response to Salmonella infection was confirmed by RT-PCR.

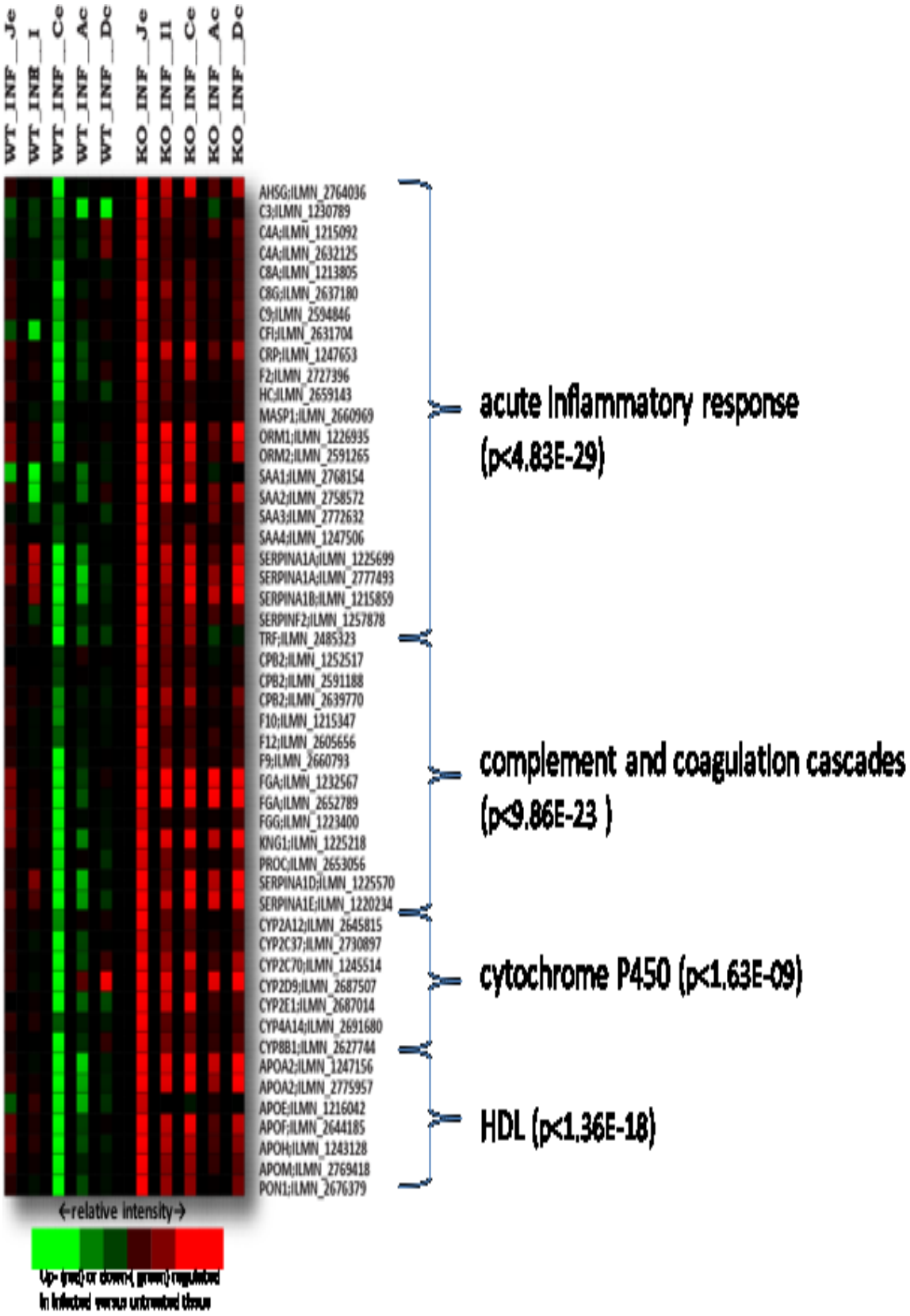
Previously we have shown that the absence of γδ T-cells in the gut epithelia changes the standard genetic profile for the mouse model that is otherwise normal. In this part of our study, we have seen that the genetic profiles of the two intestinal models, when challenged with *S. typhimurium*, are further changed by the presence of infection as well as by the absence of TCR γδ T-cells in the gut. The profile that emerges is a continued disparity between the types of functional genes up-regulated in the gut of the KO compared to WT and a marked change in the intensity of the signals generated in the KO model that is challenged by the infection. The majority of genes up-regulated at 16 hours post-infection, particularly in the jejunum of the KO, have protein products that are responsible for rapid immune response (inflammatory and coagulation pathways) and metabolic and energy pathways. In fact, the WT genes that are up-regulated in response to infection include the genes whose protein products control extra-cellular matrix processes, adhesion, and clotting in response to *S. typhimurium* infection. The question from these results is if there are phenotypic expressions of these genetic profiles, individually or acting in concert, in the absence of γδ T-cells, that measurably and significantly impair the proper functioning of the epithelial layer under the condition of infection?

Impact of the absence of TCR γδ T-cells on the GI tract Permeability during Infection.

One of our working hypothesis for γδ T-cell function is that this particular subset

FIGURE 2.3: DIFFERENTIAL GENE EXPRESSION ACROSS THE GI TRACT

Heat map of gene expression regulation (red, up-regulated, green, down-regulated) for selected annotated gene sets in response to *Salmonella* infection across all tissues of the GI tract for both WT and KO mice. The expression of these genes is highly up-regulated in the KO but not the WT jejunum and this differential up-regulation extends across multiple KO tissues.


WT_INF__Je
WT_INF__I
WT_INF__Ce
WT_INF__Ac
WT_INF__Dc
KO_INF__Je
KO_INF__I1
KO_INF__Ce
KO_INF__Ac
KO_INF__Dc
AHSG;ILMN_2764036
C3;ILMN_1230789
C4A;ILMN_1215092
C4A;ILMN_2632125
C8A;ILMN_1213805
C8G;ILMN_2637180
C9;ILMN_2594846
CFI;ILMN_2631704
CRP;ILMN_1247653
F2;ILMN_2727396
HC;ILMN_2659143
MASP1;ILMN_2660969
ORM1;ILMN_1226935
ORM2;ILMN_2591265
SAA1;ILMN_2768154
SAA2;ILMN_2758572
SAA3;ILMN_2772632
SAA4;ILMN_1247506
SERPINA1A;ILMN_1225699
SERPINA1A;ILMN_2777493
SERPINA1B;ILMN_1215859
SERPINF2;ILMN_1257878
TRF;ILMN_2485323
CPB2;ILMN_1252517
CPB2;ILMN_2591188
CPB2;ILMN_2639770
F10;ILMN_1215347
F12;ILMN_2605656
F9;ILMN_2660793
FGA;ILMN_1232567
FGA;ILMN_2652789
FGG;ILMN_1223400
KNG1;ILMN_1225218
PROC;ILMN_2653056
SERPINA1D;ILMN_1225570
SERPINA1E;ILMN_1220234
CYP2A12;ILMN_2645815
CYP2C37;ILMN_2730897
CYP2C70;ILMN_1245514
CYP2D9;ILMN_2687507
CYP2E1;ILMN_2687014
CYP4A14;ILMN_2691680
CYP8B1;ILMN_2627744
APOA2;ILMN_1247156
APOA2;ILMN_2775957
APOE;ILMN_1216042
APOF;ILMN_2644185
APOH;ILMN_1243128
APOM;ILMN_2769418
PON1;ILMN_2676379
acute Inflammatory response
(p<4.83E-29)
complement and coagulation cascades
(p<9.86E-23)
cytochrome P450 (p<1.63E-09)
HDL (p<1.36E-18)
←relative intensity→

of T-cells are responsible for the architectural and environmental maintenance of the gut epithelial layer. These functions ensure that the tissues involved in creating crucial barriers and basic functionality for the gut can be kept intact. The epithelial barriers function for the active, selective diffusion of minerals and metabolites across cell matrices. Because of this function each tissue across the GI tract has a polarity and selectively when transporting metabolites across the epithelial barrier. In Figure 2.6 the permeability of the epithelial barrier is tested using FITC-conjugated dextran substrates with a range of molecular weights ranging from 4 to 100 kD. As described in the previous chapter, TCR□□□ deficient mice have increased permeability to the low molecular weight 4kd dextran and this difference is significantly enhanced during early *S. typhimurium* insult.

In studies using the larger size dextran we demonstrated above that the wildtype and □□ T-cell deficient mouse intestines were largely impermeable to these markers. However, in the context of early *Salmonella* infection, there was an increase in permeability to all FITC-Dextran species which was strongly enhanced in the absence of □□ T cells. This suggests that while the absence of γδ T-cells causes an alteration in intestinal permeability, in the context of infection, this is exaggerated allowing even the higher molecular weight species to pass into the bloodstream. This demonstrates that while the WT intestine is undergoing significant lose, the loss of integrity is more pronounced in absence of γδ T cells.

Transmission Electron Microscopy (TEM)

Since our permeability studies reveal significant changes in intestinal

FIGURE 2.4: JEJUNAL TISSUE UNDER THE REGULATION OF γδ T-CELLS AND *S. TYPHIMURIUM*.

Super-upregulation of *S. Typhimurium* infected KO genes of the jejunum already elevated in the uninfected T-cell γδ deficient tissue. These genes include the raid immune response genes Serum Amyloid A (1-4), Serpina3m and Sepinf1/Sepinf2. In the deficient jejunal tissue, the genes whose protein products directly control the structure and function of ECM adhesion of the epithelia mucosa emerge. Those genes include elevated Claudin1 and Claudin 2 (Cldn1 and Cldn2).

	Fold Increase	p value
Serpins		
Serpina 1d	7.05	1.082E-03
Serpina 1b	4.20	1.336E-03
Serpina 1e	5.77	2.810E-02
Serpina 12	3.41	1.351E-04
Serpind1	2.60	3.810E-04
Serpina3m	2.45	1.336E-03
Serpinf	2.20	5.308E-02
ECM/Adhesion		
Laminin a3	3.43	5.717E-05
Laminin b3	2.14	3.876E-03
Tight Junctions/Desmosomes		
plakophilin4	2.20	3.244E-05
occludin	1.55	1.935E-03
desmoglein 2	1.79	5.392E-03

permeability as a consequence of infection and our working hypothesis is that an alteration in the formation of tight junctions may be the consequence of missing γδ T-cell regulation in the gut epithelia, we examined tissue from uninfected (previous chapter) and infected wild-type and TCR□□ T-cell deficient mice using TEM.

Figure 2.6 displays some of the differences observed in the gut epithelial layers of *S. typhimurium* infected WT and TCR□□ T-cell deficient mice. There are observable changes in the architectural alignment of distinct cellular compartments, as was observed to a lesser extent in the absence of infection. The tissues of the infected WT intestine still have visible compact tight junction (TJ) formation, adherens junction (AJ) formation, and well developed Desomsomes (D). In contrast, Figure 2.6d shows in the γδ T-cell deficient intestines there are subtle differences surrounding the formation of the tight junction (not as compact as the WT counterpart) and smaller adherens formations, and poorly defined desmosomes.

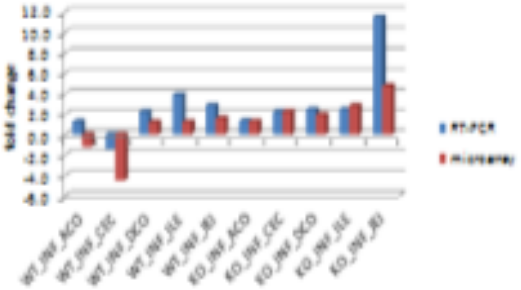
DISCUSSION

It is clear from the data presented here that the transcriptional program in γδ T-cell KO mice has been profoundly and specifically altered across the multiple tissues of the GI tract. At baseline there has been a decrease in energy metabolism and an increase in cell cycle related genes. It is evident that the removal of the γδ T-cell from the gut environment leaves a unique biological footprint which, at the very least, is implicated in pathogenic processes and host response to infection. Significant adverse effects of γδ T-cell deficiency to normal GI function such as the changes in membrane permeability

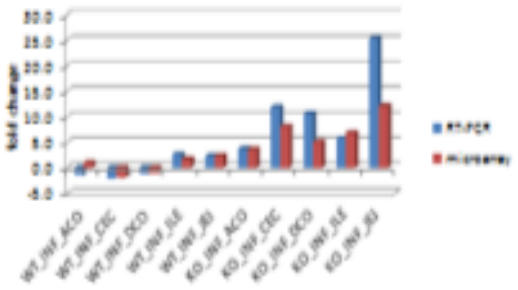
Figure 2.5: Gene Expression Microarray Results Validated by RT-PCR

Individual inflammatory gene expression microarray results were validated by RT-PCR for both WT and γδ T-cell deficient GI tissues (liver excluded). Gene expression regulation is significantly elevated by both microarray and RT-PCR for deficient tissue but not WT jejunum.

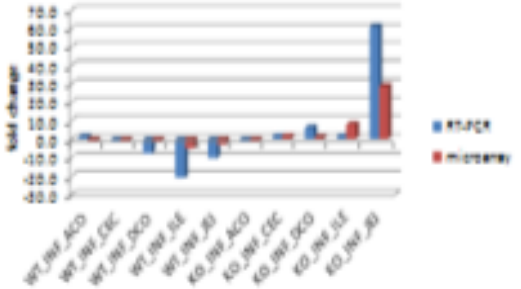
mu_ApoC1



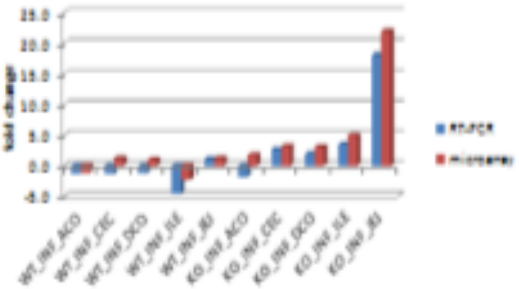
mu_Hp



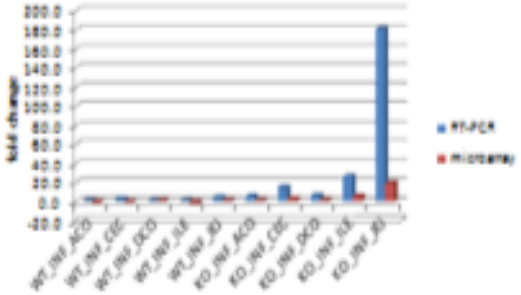
mu_Saa1



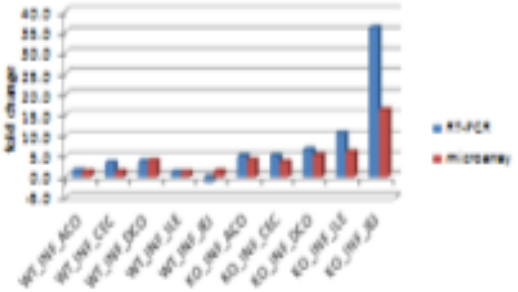
mu_Lcn2



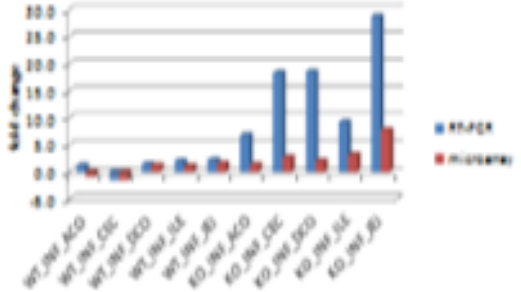
mu_Saa2



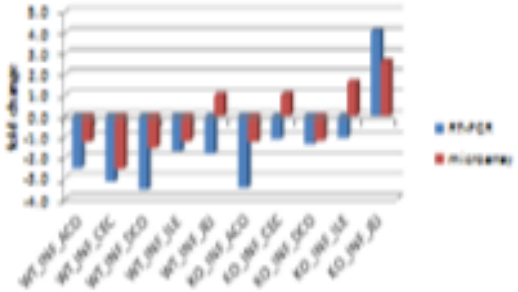
mu_Serpina 3n



mu_Serpina 3m



mu_Serpina f1



suggests in that some level of significant disruption must occur.

Alternatively, increased intestinal membrane permeability of the γδ T-cell KO in response to infection may lead to robust *Salmonella typhimurium* bacteria breakthrough into the circulatory system, leading to a chronic cycle of systemic inflammation. What is novel from this study is transcription of key acute phase genes such as SAA 1-4, and multiple SERPIN genes takes place in the absence of γδ T-cells directly in the intestinal tissue of KO mice. However, there is little or no evidence for the transcription of pro-inflammatory cytokines in either WT or KO intestinal tissues. Since the acute phase response is a part of the innate immune response preceding specific immune responses and acts transiently as a critical component in the early establishment of host defense, the chronic nature of its presentation in KO mice may also suggest a negative regulatory role of γδ T-cell in controlling the inflammation process in the gut. Conversely, the presence of prolonged acute phase gene expression in intestinal tissues may indicate the exertion of a protective effect associated with the acute phase proteins themselves.

Serum Amyloid A (Saa1/Saa2) is a gene family of interest in the rapid immune response that is super-upregulated by infection in the KO jejunum. SAA are a family of apolipoproteins that are secreted during the host's rapid immune response, or, more precisely in the moment of inflammation. These proteins have several roles, including the transport of cholesterol to the liver for secretion into the bile, the recruitment of immune cells to inflammatory sites, and the induction of enzymes that degrade extracellular matrix. SAAs are reported in several chronic inflammatory diseases including the autoimmune disease rheumatoid arthritis.[92]

During the inflammatory process both Saa1 and Saa2 are expressed and induced principally in the liver are secreted to affected areas of the body. SAA1 and SAA2 genes are regulated in liver cells by the pro-inflammatory cytokines IL-1, IL-6, and Tumor Necrosis Factor-α (TNF-α). These cytokines also have prominent functions in complement immune pathway regulation and coagulation cascades. Both Saa1 and Saa2 are induced up to a 1000-fold in mice under acute inflammatory conditions following exposure to bacterial lipopolysaccharide (LPS), which is the major surface membrane molecule present in almost all Gram-negative bacteria (such as *S. typhimurium*) and act as stimulators of innate or rapid immunity in many eukaryotic species.

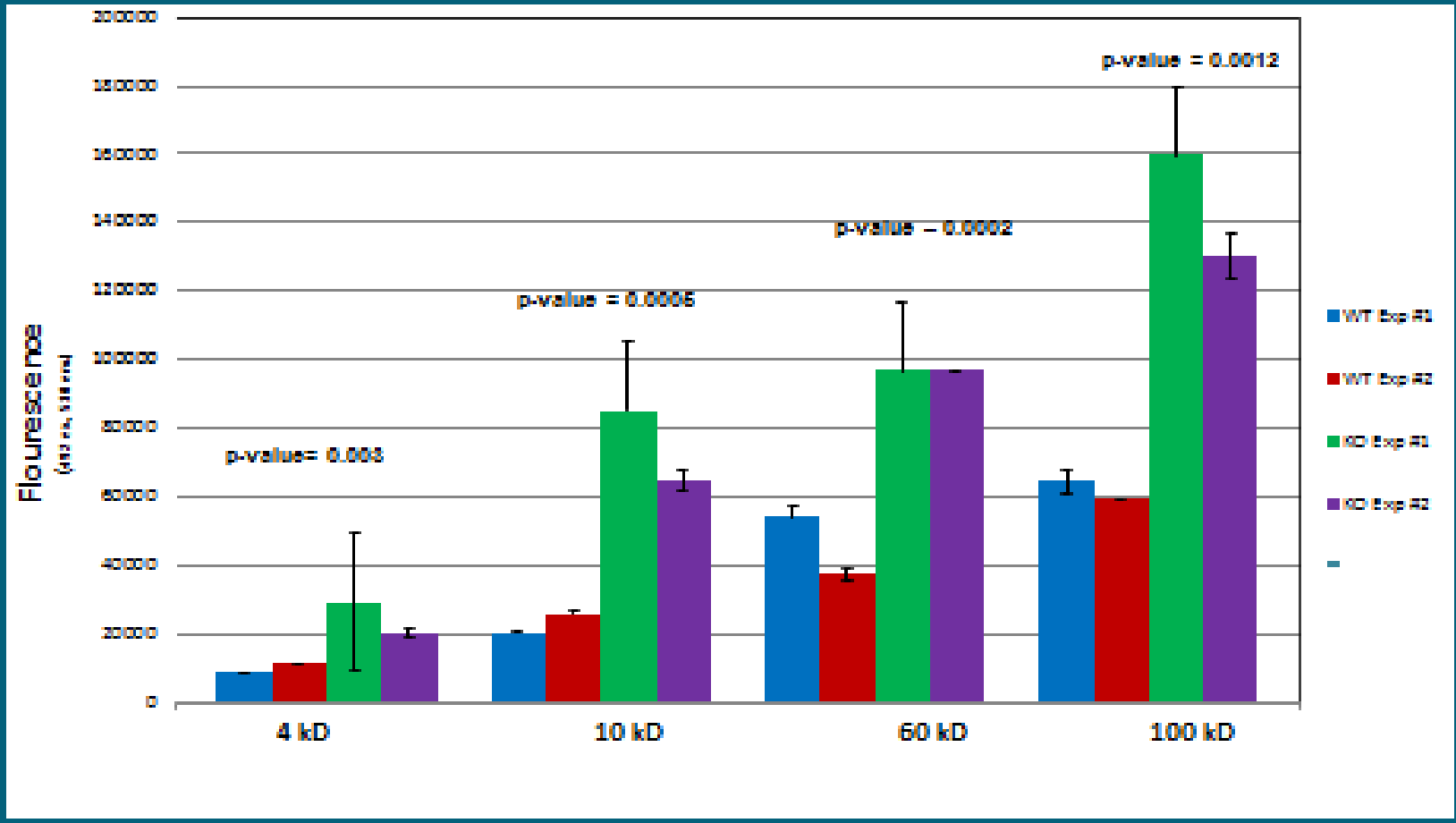
Another family of genes upregulated prominently in the jejunum of TCR γδ deficient intestines in acute inflammatory response includes Serpina1. This member of the Serpin gene family is part of gene isoform shown to be significantly up-regulated in the jejunum of uninfected γδ T-cell deficient intestines. These Serpins are remarkable for the study of human disease because they are murine functional homologues to human gene SERPINA1.[93,94,95,96] The protein encoded by this gene is secreted and is a serine protease inhibitor whose targets include elastase, which is a primary target; plasmin and thrombin for which it has a moderate infinity; and trypsin, chymotrypsin, and plasminogen activator, which Serpina1 binds irreversibly.[97,98,99] The aberrant form inhibits insulin-induced NO synthesis in platelets, decreases coagulation time and has proteolytic activity against insulin and plasmin.[100]

The super-upregulation of Serpin gene family members and those of genes whose products are responsible for the acute, rapid immune response to infection in the jejunal

FIGURE 2.6: COMPROMISED INTESTINAL INTEGRITY *S. TYPHIMURIUM* INFECTION IN THE ABSENCE OF TCR γδ T-CELLS.

Age-matched wildtype and γδ T-cell deficient mice were orally infected with 5 x 10^7 CFU/ml of *S. typhimurium* and orally infected with 2.2mg/ml of varying molecular weights of FITC-Dextran Serum was isolated 16 hours later and the FITC-Dextran signal was detected spectrophotometrically at 492 nm. The permeability of the epithelial barrier is shown using FITC-Dextran a) 4KD, b) 10 KD, c) 60 KD, and d) 100KD.

Effects of *Salmonella typhimurium* Infection on Intestinal Permeability



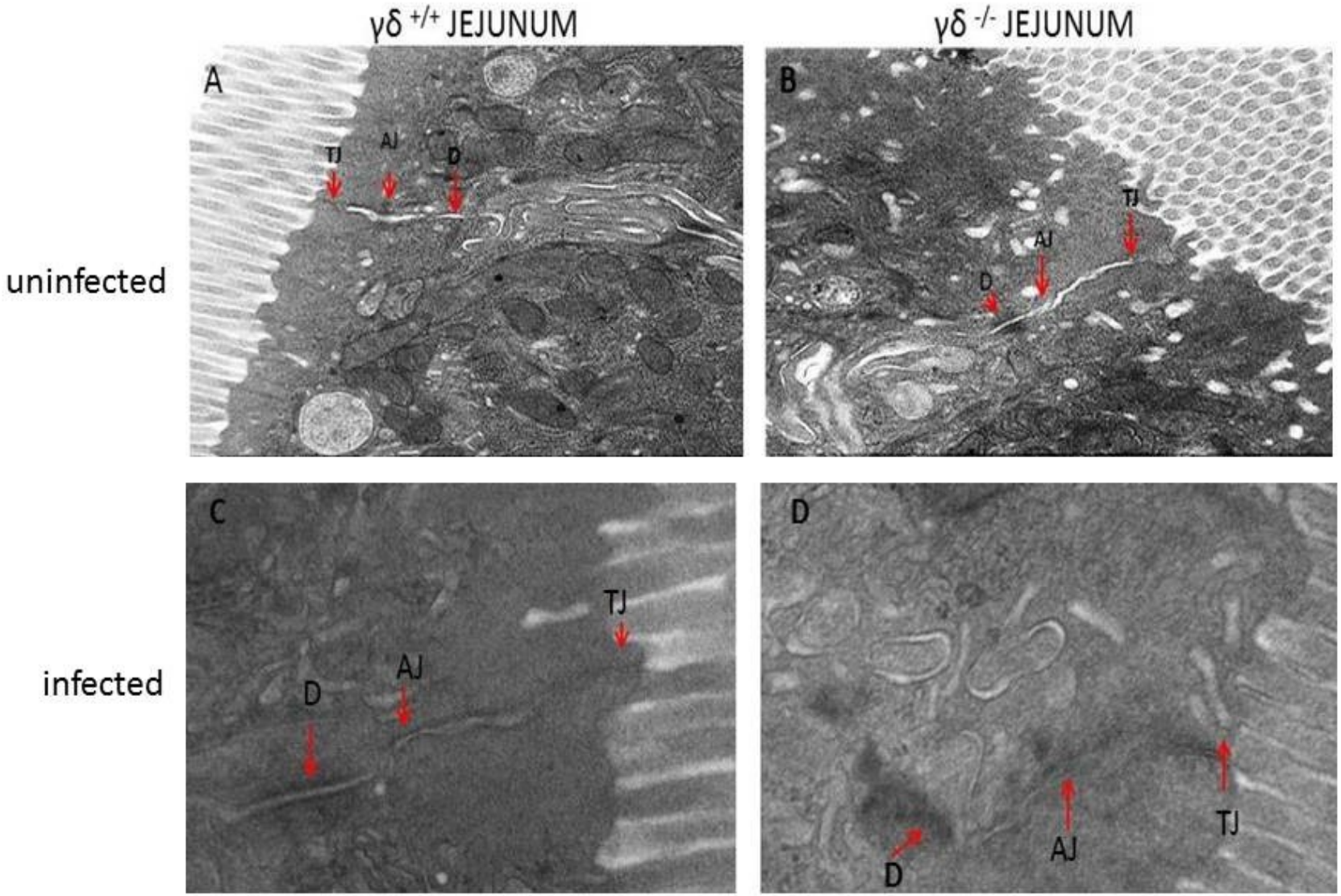
tissue of the small intestine could be predicted from the baseline up-regulation of these genes in uninfected TCR□□ T-cell deficient mice. Although the jejunum's role is normally associated with absorption and digestion of macromolecules in the gut, in the uninfected TCR□□□ deficient mice it is at the center of an acute, primary immune response. This function is not normally associated with this tissue type, but the absence of the γδ T-cell population in this epithelial layer seems to correlate with this tissue functioning outside the parameters of its typical role.

The elevated regulation of genes such as SerpinF1 and the Claudins, including Claudin 1 and Claudin 2, in the jejunum of those mice without TCR γδ T-cells (Figure 2.3) shows there is a modeling/remodeling and structural maintenance role of these T-cells in the gut epithelia. The protein product of SerpinF1, also known as pigment epithelium-derived factor (PEDF), is a secreted protein that is a non-inhibitory neurotrophic factor, and an angiogenesis and tumorigenesis regulator.[101, 102, 103, 104] In the absence of TCR γδ T-cells, the gut up-regulates genes having a regulatory or direct role in vascularization and clotting. All of these functions are important in getting immune cells to the location of infection. The neo-vasculature and the regulation of blood flow are also crucial components of tissue repair after biological insult and injury. However, even with the up-regulation of these genes and the functioning of their protein products, the sustained tissue damage in the wake of infection proves that even the protein products of these genes fail to be an adequate substitute for the γδ T-cell for tissue repair and immune response in the gut.

The emergence the Claudins in the infected γδT-cell KO's in response to infection

FIGURE 2.7: THE JUNCTIONAL COMPLEX DEVELOPMENT IN THE INTESTINAL JEJUNUM UNDER *S. TYPHIMURIUM* INFECTION

Cross-sections of jejunum tissue of a) normal WT; b) TCR γδ deficient, uninfected; c) infected WT with tight junction (TJ), Adhesion junctions (AJ), and Desomsomes (D) shown: and d) TCR γδ T-cell deficient, infected with tight junction (TJ), Adhesion junctions (AJ), and Desomsomes (D) shown.


γδ +/+ JEJUNUM
γδ -/- JEJUNUM
uninfected
infected
A
B
C
D
TJ
AJ
D
TJ
AJ
D
D
AJ
TJ
TJ
AJ
D

is important in possibly explaining the type of structural, architectural, and functional regulation provided by these T-cells in the gut. The Claudins are more directly involved in the maintenance and functioning the extra-cellular matrix (ECM). Claudins are 18- to 27-kD tetraspan proteins containing a short cytoplasmic N-terminus, two extracellular loops and a C-terminus cytoplasmic domain.[105] Claudins do not share any sequence similarity to occludins, whose protein also localize to tight junctions.[106] Claudins are capable of forming tight junction strands and, therefore, form the backbone of the tight junction.[107] The morphological and physiological properties of tight junctions are determined by the combination and ratios of Claudin species.[108] It is also important to note that a well-functioning epithelial barrier is dependent on the presence of tight junctions.

It can be inferred that without the presence of γδ T-cells in the gut the maintenance and proper functioning of tight junction, and therefore, the entire gut epithelia is deficient. These junctions keep the integrity of the tissue and makes sure that it remains a protective barrier and regulator of molecules across the epithelia. Thus, the proper maintaining of the gut environment protects the bloodstream, circulatory system, and ultimately, the host from disease progression. In the absence of γδ T-cells, the Claudins alone prove lacking in their ability retain the protective barrier and homeostasis for epithelia ECM that is demonstrated with our permeability studies and structural analysis of TEM.

Permeability of Mucosal Epithelia

One of our working hypothesis for γδ T-cell function is that this particular subset

of T-cells are responsible for the architectural and environmental maintenance of the gut epithelial layer. Where there is the proper functioning of the gut epithelial layer we believe that the intestines are better positioned to withstand a biological infection. These functions ensure that the tissues involved in creating crucial barriers and basic functionality for the gut can be critical for the survival of the host against biological infection or injury. We have shown in this section that the absence of TCR γδ T-cells in the gut epithelia changes the standard genetic profile of the GI-tract under biological insult from *Salmonella typhimurium*. In the previous sections chapter we have also shown that the gut epithelial layer without injury or infection, but also lacking the crucial TCR γδ T-cell population, is already architectural compromised, particularly in the formation of their tight junctions. The integrity the intestinal membranes of those uninfected knockout mice were also severely compromised.

Therefore, even when the intestinal wall begins in a state of homeostatic maintenance and functionality, a large biological challenge as represented by the *S. typhimurium* infection can cause significant damage and dysfunction in in the barrier. This dysregulation and dysfunction can possibly lead to chronic inflammation cycles or fatal harm to the host when faced with biological insult.

The overwhelming loss of permeability function as seen in the gut epithelial of those mice without the TCR γδ T-cell population underscores this population's role in regulating and maintaining the intestinal epithelial layer for proper functioning of the intestines for molecules diffusion and balance, as well as, for a the structural integrity to help the host fight disease and injury.

TEM of Gut Epithelial Layer

The intestinal tissue architecture functions to provide a regulated exchange of nutrients, macromolecules, and gases from the intestinal lumen to the bloodstream. From the brush border of the apical (or luman) side molecules first encounter tight junctions. Through the process of receptor-mediated endocytosis the tight junctions will selectively allow molecules to be actively transported from the apical villus to the epithelial layer of the tissue. Larger molecules and particles (pathogens) are however actively excluded from penetration. Once the molecule transverses the epithelial layer, tight junctions carry on exocytosis to deliver nutrients and molecules on to the bloodstream. The well-developed architecture of the epithelial layer underscores the regulated functioning of the normal WT gut where there is no or minimal detection of FITC-Dextran leakage into the bloodstream. Therefore, along with the well-regulated architecture in the presence of TCR γδ, there is a well-regulated functioning of the gut epithelial layer.

Our working hypothesis is that an alteration in the formation of tight junctions may be the consequence of missing γδ TCR regulation in the gut epithelia. We have observed using TEM that infected wildtype gut epithelial tissue from the jejunum show visible damage. There is observable morphology showing poorly-developed columnar intestinal epithelial cells and a lack of demarcation in the apical and baso-lateral sides of the intestinal tissue in the infected jejunum.

In infected γδ T-cell deficient tissue, there are distinct differences in its morphology and architecture even compared to the infected WT. The infected γδ T-cell deficient tissue shows slightly less junctional definition formation (tight junction, adhesion junction, and desmosomes) compared to the infected WT and the uninfected intestinal tissue. There is a pronounced lack of tight junction definition across the tissue, with no visible adheren junctions, and reduced size desmosomes.

When coupled with the results from the permeability tests, which showed low barrier function in the γδ T-cell deficient gut, the intestines of infected KO are decimated in the aftermath of biological insult. The architectural and functional changes seen in the infected KO gut underscores that the lack of function at this point in the infection may be due to the tissue encountering, but, not effectively being able to respond to *S. typhimurium* infection. The structural changes seen in the infected γδ T-cell deficient gut with the infected WT tissue emphasizes the difficulty this tissue has in responding to infection induced injury in the absence of γδ T-cells.

The subtle differences in the defined junctions in the γδ T-cell deficient host can cause dysfunction in the regulation of molecules and promote dissemination of S. *typhimurium* from the GI tract into the bloodstream. These results indicate that this particular iIEL population may directly affect the maintenance and regulation of gut epithelial function by controlling the development and/or presence of the crucial tissue structures such as tight junctions for the proper functioning of intestinal epithelium. These results indicate that this tissue maintenance, which is absent in the removal of γδ T-cells from the gut, is important for the tissue to be able to effectively respond to biological challenge. These findings help define the role of γδ T-cells in aiding in immune defense because of its role in tissue maintenance.

CONCLUSION

Deletion of γδ T-cells from the environment of the gut has large phenotypic consequences for gene transcription, structural architecture and maintenance, and functionality in the wake of pathogenic challenge. Functional annotation analysis of the genes differentially regulated in the absence of TCR γδ T-cells reveals a very significant

enrichment for genes of the acute phase response. This response is both specific to the γδ T-cell deficient mouse jejunum and is already elevated at baseline and become more amplified following *S. typhimurium* infection.

The super-upregulation of genes already elevated in the absence of infection such as Serpina1 and Serum amyloids underscores just how impaired the baseline of the TCR γδ T-cells deficient intestine started. The γδ T-cell deficient intestine, already under the duress of lacking this key subset, has to have these genes upregulated as part of normal homeostasis. In the time of biological insult, this compromised tissue has to use these pathways of regulation as a source to fight against infection. However, these results may indicate that the compromised tissue has a much harder time against *S. typhimurium* infection in its already compromised state.

The proper maintaining of the gut environment protects the bloodstream circulatory system and ultimately the host from disease progression. In the absence of TCR γδ T-cells, the Claudins, either through the lack of T-cell regulation, or in a more substantial role than they usually have in the presence of TCR γδ T-cells, are inadequate in providing the structural and functional support of the gut functioning against infection. It can be inferred that without the presence of γδ T-cells in the gut the maintenance and proper functioning of tight junction, and therefore, the entire gut epithelia is deficient. Thus, the proper maintaining through the role of TCR γδ T-cells of the gut environment protects the blood, circulatory system, and, ultimately, the host from disease progression.

These results indicate that this particular iIEL population may directly affect the maintenance and regulation of gut epithelial function by controlling the development

and/or presence of the crucial tissues such as tight junctions for the proper functioning of intestinal epithelium. TCR γδ T-cells are crucial in a regulatory role for hemostasis of the gut environment, which in turn, ensures proper tissue functioning and is the foundation of a robust immune response to infection and injury.

CHAPTER 3

The Effects of the TCR γδ Deficiency in the GI and the Systemic Changes Effecting Liver Tissue

INTRODUCTION

The liver is a peritoneal tissue responsible for ridding the body of toxins. Its proximity to the GI tract makes the liver tissue suitable for comparing and contrasting its response to *Salmonella typhimurium.* The liver is also the first tissue with which to determine the extent and timing of the systemic damage done to the host when there is a catastrophic genetic alternation in GI. This change in the genetic profile is first observed when the T-cell γδ is deleted from the gut mucosa. This deletion starts various changes in the genetic and phenotypic characteristics of the tissue. This change in the composition of the gut mucosa, in regards to its TCR γδ T-cell population, has implications for the function of the neighboring liver tissue when it comes to response to infection and progression of the disease state in the tissue. When there is that type of failure in an important tissue such as the GI, with its many roles, there are broader consequences for other tissues within close proximity. Therefore, we attempted to the use the liver tissue to elucidate the systemic and far reaching results of failure of functionality in the GI due to the loss T-cell γδ.

The Liver Tissue

Hepatocytes are the main functional cells of the liver. These hepatocytes are connected through a network of connective tissue that forms branches and extends outward into the liver cytosol, or parenchyma. This connective tissue architecture provides a scaffold to support the organ, which lacks the collagen-and fibril-based connective tissue. This organization also provides a way for blood vessels, lymphatic vessels and bile ducts to carry blood, lymph, and bile to the liver. Additionally, the sheets of connective tissue divide the parenchyma (cytosol) of the liver into very small

units called lobules. On the distal location of the lobules are orderly distributed portal triads, which contain a bile duct, a terminal branch of the hepatic artery, and portal vein. Hepatocyte nuclei are distinctly round in shape, with one or two prominent nucleoli.

These hepatocytes are responsible for synthesis of most important plasma proteins found in the liver. One protein is albumin, the major plasma protein that is synthesized almost exclusively by the liver. The liver produces globin, one of the two components that form hemoglobin, which is a substance that carries oxygen in red blood cells. A certain number of globulins, which are proteins that includes antibodies for rapid response immunity and acquired immunity, are produced in the liver. Also, the liver synthesizes many of the clotting factors necessary for blood coagulation, a process that helps wounds to heal by enabling blood to clot.

A unique feature of the liver is that it has several anatomical compartments for leukocyte recruitment, including the endothelial cells lining hepatic sinusoids, and the endothelial cells lining the portal and terminal hepatic veins.[109,110] Leukocytes can adhere and migrate across the hepatic microvasculature. However, but the majority of these cells enter the parenchyma via the hepatic sinusoids.[111] The endothelial cells lining the hepatic sinusoids have distinct characteristics that are unlike the neighboring GI tract. Among them is the absence of an underlying basement membranes and tight junctions. Instead they contain fenestra, a small pore in endothelial cells that allow for rapid exchange of molecules between sinusoid blood vessels and surrounding tissue.[112] Adhesion molecules such as E- and P-selectin, which are expressed on endothelial surfaces of hepatic arteries, portal and central veins are absent in sinusoidal endothelial cells.[113, 114] The liver is host to a very important part of the phagocytic system. Embedded in the sinusoids are large amounts of Kupffer cells, a type of liver tissue

macrophage. Kupffer cells are actively phagocytic and represent the main cellular system for removal of particulates and microbes from the circulation. Their location downstream of the portal vein allows Kupffer cells to efficiently seek and capture bacteria that get into portal venous blood through breaches of the intestinal epithelium, and, thus preventing invasion of the circulatory system.

METHODS

Tissue Isolation

The hepatic tissues of both the WT and TCR γδ T-cell deficient mice were rapidly dissected from sacrificed mice and perfused in ice-cold 1X PBS 16 hours after an orally administered a mock dose of sterile 1M PBS or infected orally with 5×10^8 CFU aroA− CFU *Salmonella* in 0.1 ml of PBS.

In vivo infection with S. typhimurium

Eight- to 10-wk-old gender-matched mice were either mock infected with sterile PBS or infected orally with 5×10^8 CFU *Salmonella typhimurium* in 0.1 ml of PBS, using a standard gastric intubation needle. At various times after infection, the liver isolated from infected and control animals. The number of bacteria present in each mouse strain was determined by preparing single-cell suspensions from the mesenteric lymph nodes and spleen. Suspensions were grown on TS agar plates.

Immunohistochemistry

The mice sections from livers were cut from sacrificed mice. The tissue was not perfused. A 2.0 mm section of the left lobule was removed and rapidly fixed over 48 hour period at room temperature in freshly prepared fixative solution (formaldehyde 4%

w/v, 0.1 M phosphate buffer, pH 7.2). The tissue is dehydrated through a series of ethanol gradients. The tissue is then fixed in wax blocks. The paraffin embedding procedure was performed by Johns Hopkins Surgical Histology Laboratory.

Tissue Blocking and Staining

Sections 5 μm thick were cut from the paraffin block using a microtome. A de-paraffinizing technique was used to rehydrate the tissue through a series of decreasing ethanol gradients until it was finally immersed in 50% ethanol. Antigen retrieval was performed using 0.01M citrate buffer pH 6.0, and endogenous peroxidase activity was quenched by a hydrogen peroxide 1% solution. For the liver sections, endogenous biotin action was blocked in a 0.001% Avidin/0.001% Biotin Solution.

For H&E staining, slides were immersed in Gill 1-Regular Strength Hematoxylin (Ricca Chemical Company, 3535-16) for 5-10 minutes. The slides were washed over tap water and 1% acid alcohol solution. Eosin Y was added to slides for contrast. The slides were mounted with the Permount (Fisher Scientific, SP15-100).

Macrophage and Neutrophil Staining

Sections were incubated with rat anti-macrophage F4/80, anti-macrophage BM8, (Abcam, ab16911); and rat, anti-neutrophil NIMP-R14 (Abcam, 551776). Samples were treated with a secondary antibody, goat anti-rat IgG (H+L)-Biotin, (Southern Biotech, 3050-08). Detection was with horseradish peroxidase-streptavidin-biotin complex supplied by Vecta-Stain Elite ABC (Vector Labs, PK-6101). Sections were then counterstained with Gil 1 hematoxylin.

Tissue Isolation for Microarray

Details are located in Chapter 2.

In vivo infection with S. typhimurium for Microarray

Details are located in Chapter 2.

RNA Extraction

Details are located in Chapter 1.

Microarray Analysis

Details are located in Chapter 1.

Analytical Methods and Statistical Analysis

Details are located in Chapter 1.

RESULTS

Histopathology

Comparison of Uninfected Normal Liver to Uninfected γδ Deficient Liver

In the H&E staining observed in Figure 3.1a and 3.1b, both the WT and *γδ* deficient liver show similar structural and cellular features. Both mouse liver phenotypes have many normal, characteristic channels that coalesce to form a large network of hepatocytes found in healthy liver. Healthy hepatocyte nuclei are seen in both Figures 3.1a and 3.1b. Figures 3.1a and 3.1b show normal hepatic tissue structure with parenchyma separated by vascular sinusoids and visible lobules in a normal hexagonal structure.

Comparison of Liver Responses to S. typhimurium Infection

Ten WT and ten γδ T-cell deficient mice were administered $5x10^8$ CFU of *S. typhimurium* in 0.1 ml. doses. We observed the liver pathology and its progression in

both strains at days 1, 5, and 10 (days post infection, dpi). No observable pathology can be seen at day one (figure not shown).

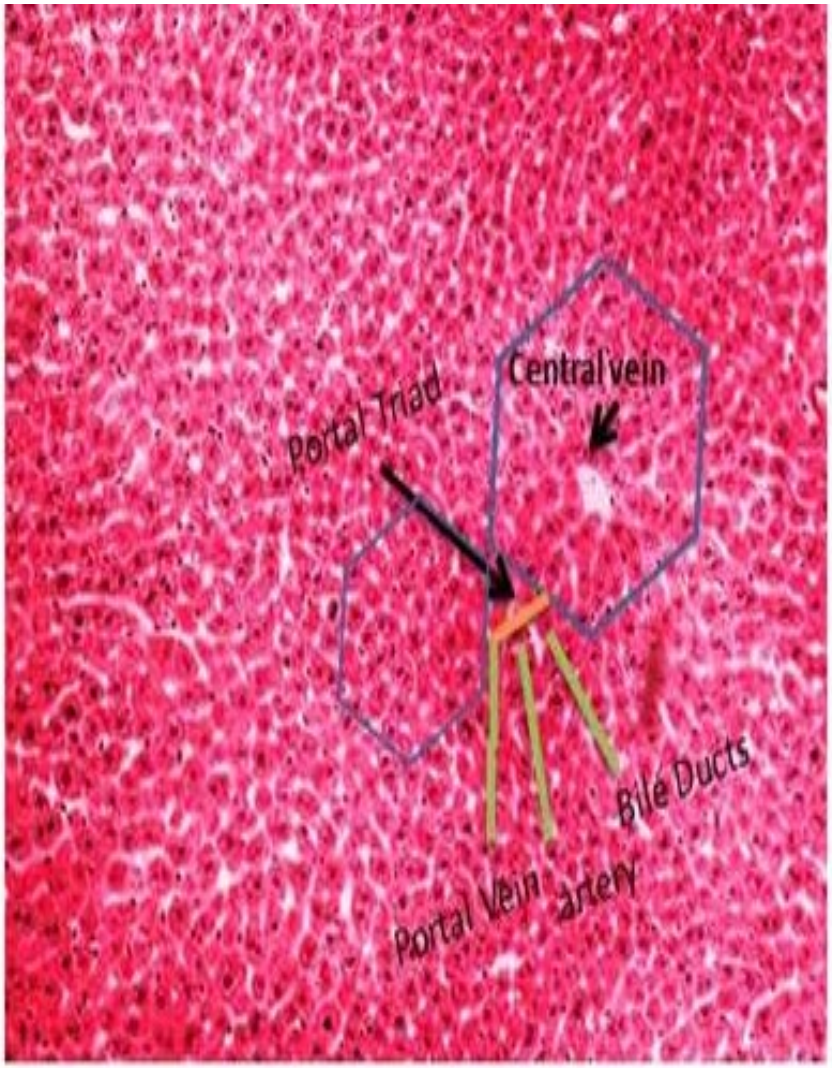
There are distinct changes detected at 5 dpi. The infected WT has neutrophil staining seen both in the H&E stain and the "saddle-shape" or crescent morphology of the cell in Figure 3.2a. However, the influx of cells does not overwhelm the tissue since the WT liver does not sustain structural damage with intact portal veins and ducts. In the infected γδ T-cell deficient liver shown in Figure 3.2b, we observed the entire parenchyma punctuated by a large influx of neutrophils that permeate the portal veins and ducts. Figure 3.2d further confirms that the cell stained and detected in Figure 3.2b are neutrophils because the neutrophil- specific antibody NIMP-R14 stains these cellular bodies.

In Figure 3.3 the liver macrophage cells have been specifically stained to determine the progression of pathology in the γδ T-cell deficient and WT livers. At 5 dpi the WT liver shows a pronounced lack of staining against the F4/80 anti-macrophage and against the anti-neutrophil NIMP-14, as seen in Figure 3.3a. There are numerous nuclei in the parenchyma and no immune cell infiltration in the portal veins or ducts. However, in Figure 3.3b, the anti-macrophage F4/80 antibody staining shows a very concentrated population of macrophages that surround the neutrophils in the infected γδ T-cell deficient tissue.

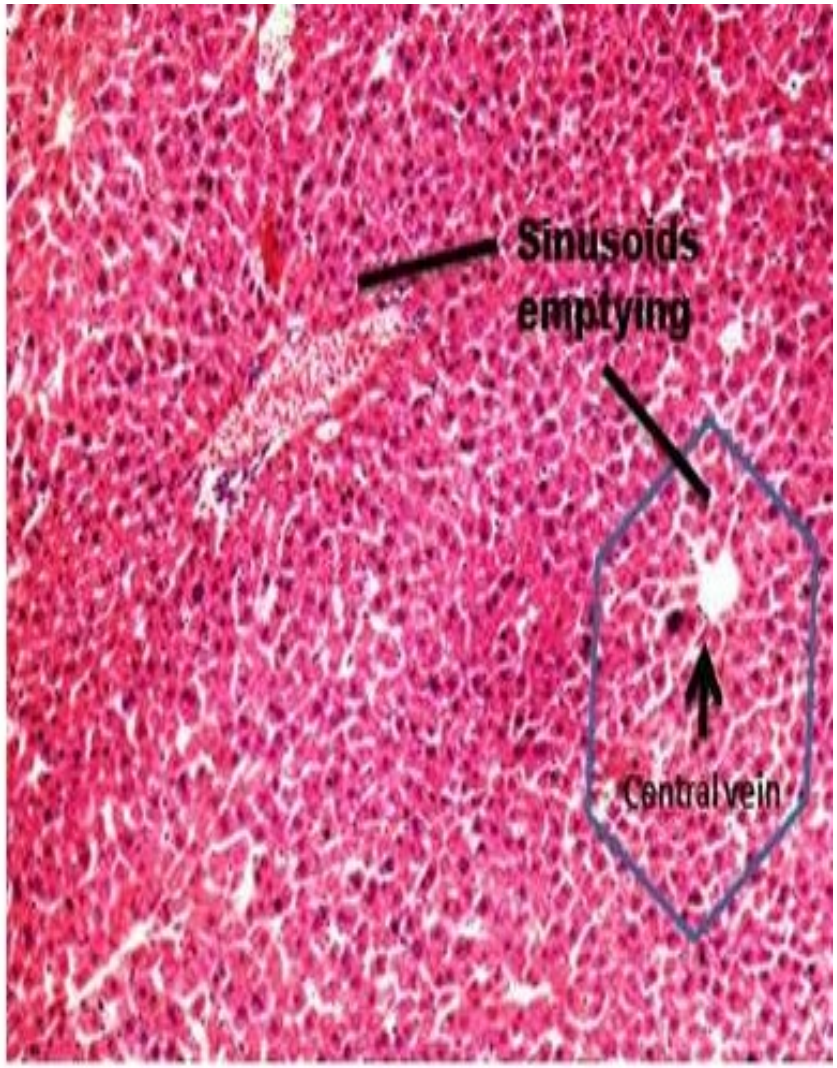
The most distinct signs of pathology are seen in staining the TCR γδ T-cell KO

FIGURE 3.1: COMPARISON OF NORMAL WT AND γδ T-CELL DEFICIENT LIVER TISSUES

Shown is the histology of normal tissue compared to γδ T-cell deficient liver sections using hematoxylin and eosin staining (10x mag). A) Normal, WT hepatocyte with parenchyma (cytosol) separated by vascular sinusoids, visible lobules in a hexagonal structure. No remarkable morphological deformities; B) Uninfected, γδ T-cell deficient hepatocyte with parenchyma separated by vascular sinusoids, visible lobules in a hexagonal structure. No remarkable morphological deformities.



A) WT Uninfected Normal Liver Tissue, H&E Stain, (10x)



B) KO Uninfected Liver Tissue, H&E Stain, (10x)

liver at 10 dpi. In Figure 3.4b, the entire parenchyma in the deficient tissue is overwhelmed with neutrophils. The portal veins and ducts are no longer visible. There are dispersals of red around the remnants of veins indicating thrombosis in this KO liver. There is no observable tissue structure, which have a high amount of neutrophil influx that has not been cleared by macrophage. There is considerable inflammation and complete absence of the structural arrangement. The parenchyma has coalesced and sclerosis is evident. Fibrosis and collagenation is present in a tissue that, if healthy, is virtually devoid of these structures. This demonstrates that a complete necrosis in this tissue has occurred. The WT also has an influx of neutrophils as seen by the blue staining and morphology of the neutrophilic cell in Figure 3.4a. There is evidence that sclerosis is beginning in the parenchyma. However thrombosis and necrosis is not seen.

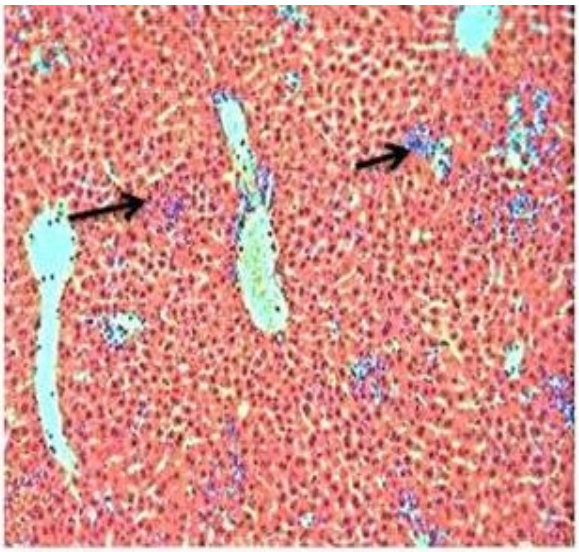
Transcriptome Analysis

In Figure 3.5, the heat map of the liver and GI tract show the global expression of genes regulated by the deletion of the TCR γδ T-cells and by the introduction of the *S. typhimurium* infection. The zoom and boxed area of the heat map shows a high degree of correlation between the genes up-regulated in the KO liver and the genes with elevated regulation in the lower GI tract of the liver. Specific pathways upregulated in KO tissue include complement and coagulation cascades (DAVID, $p < 9.9\text{-}23$), cytochrome P450 (DAVID, $p < 1.6\text{-}09$), and HDL (DAVID, $p < 1.4\text{-}18$) pathways.

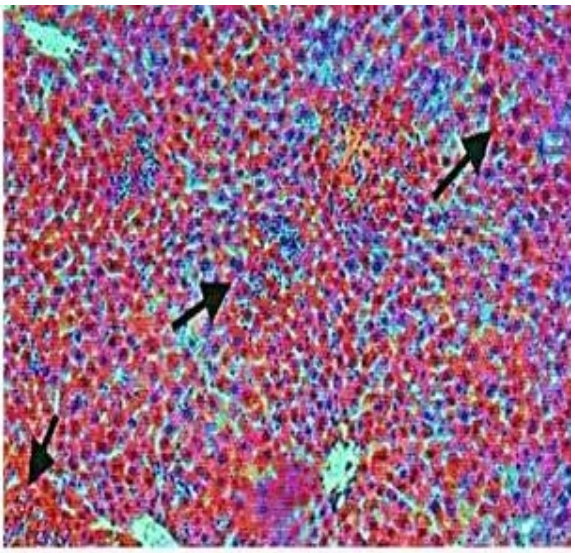
An interesting finding is that there is a very close correlation between the livers of both WT and KO. Figure 3.6 shows the gene expression of a sizeable overlap (35% for WT, 40 % for KO) of statistically significant DEG's in response to infection in liver for both mouse phenotypes. By comparison the average overlap of DEGs for all the tissues

FIGURE 3.2: IMMUNOPATHOLOGY OF WT VERSUS γδ T-CELL DEFICIENT LIVERS

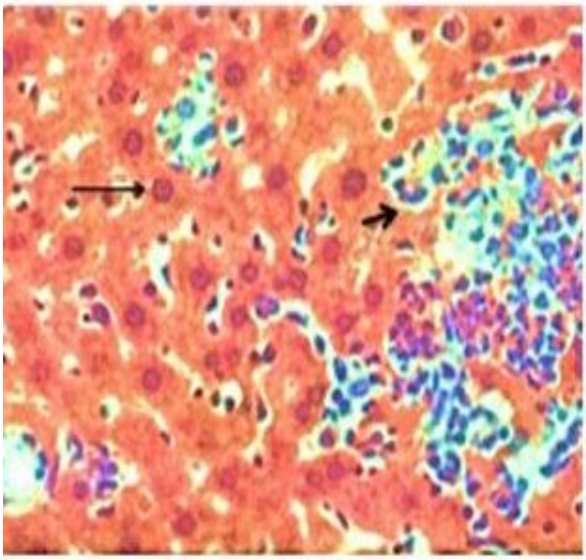
H&E and antibody staining of livers 5 dpi with $5.0x10^7$ CFU/ml. showing: A) clusters of neutrophilic bodies stained blue in the infected liver section (10x mag); B) neutrophilic bodies stained blue have engulfed the infected liver tissue of the γδ T-cell KO mouse, and the parenchyma is obscured by the influx of neutrophils that have infiltrated the portal veins and ducts (20x mag.); C) morphologically the round bodies represents the nucleus of the hepatocytes as opposed to the "saddle-like" or crescent shaped neutrophils which is seen (40x mag.); and D) NIMP-14 anti-neutrophil/ NovaRed signal detection shows the intense influx of neutrophils at 5



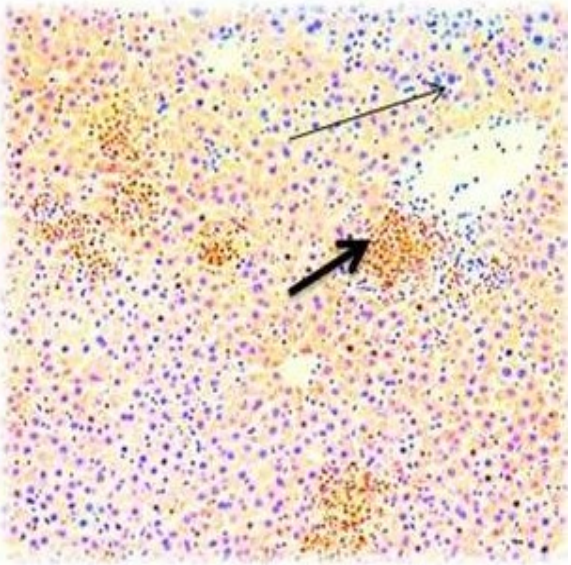
A) WT Liver 5 days post infection (10x) H&E Stain



B) KO Liver 5 days post infection (20x) H&E Stain



C) H&E Stain of KO Liver (40x) : The morphological distinction between neutrophilic and nucleic bodies



D) KO Liver 5 days post infection (10x) NIMP-14 Clone Anti-neutrophil; Vector NovaRed Dection; Hemotoxylin Couneterstain for Nuclei

of the GI tract, not including liver, was just 12% for WT and 12.6% for KO. The overlap of these genes between WT liver only with KO GI tract and KO liver is well illustrated in Figure 3.5, a heat map of all statistically significant differentially regulated genes derived across all tissues. The overlapping genes in liver specifically enriched for the acute inflammatory response (DAVID, $p < 8.5\text{-}13$).

Figure 3.7 shows that genes differentially up-regulated in the γδ T-cell deficient liver include those of the ECM/Adhesion genes Lamini4, Actn4, Cldn1. These genes show a fold change above 2.0 and p-values ≤ 0.05.

DISCUSSION

Comparison of Uninfected Normal Liver to Uninfected γδ Deficient Liver

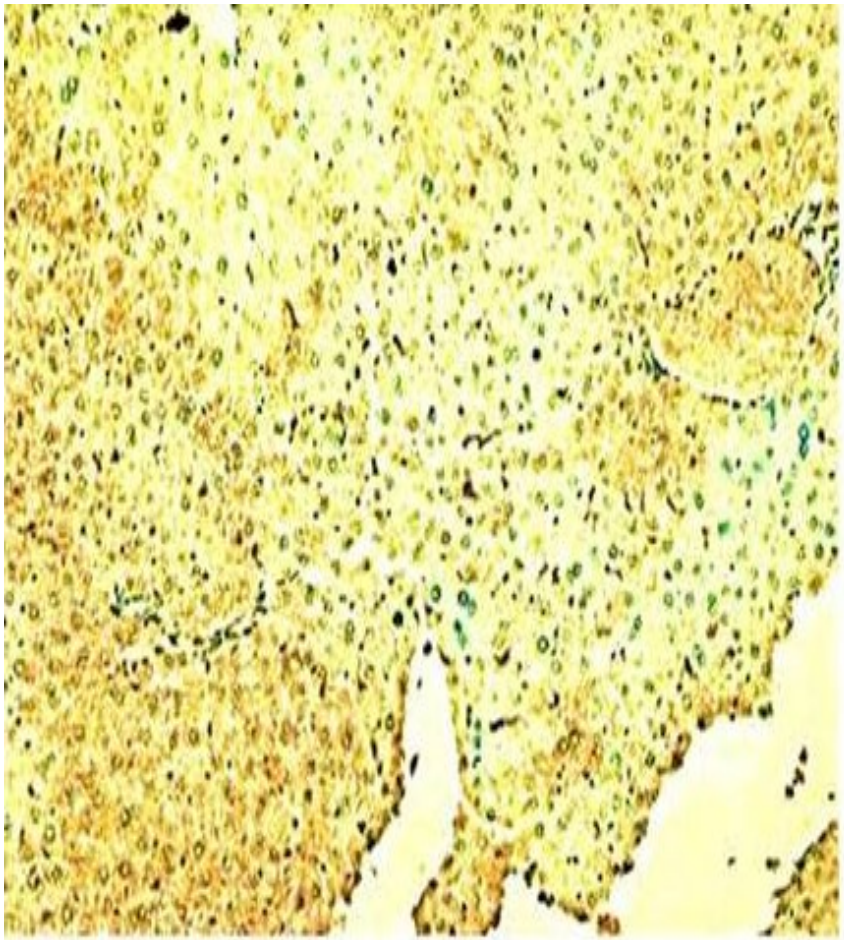
In normal tissue portal tracts have a triangular or round morphological structure that have the pre-terminal and terminal portal veins, terminal branches of hepatic artery, and bile ducts which are all rooted in fibrous connective tissue. Except for the presence of some lymphocytes such as the resident Kupffer cells, healthy tissues have no presence of inflammatory cells or effector molecules in the portal tracts. As shown in KO liver in their non-infected state.

Comparison of Liver Responses to S. typhimurium Infection

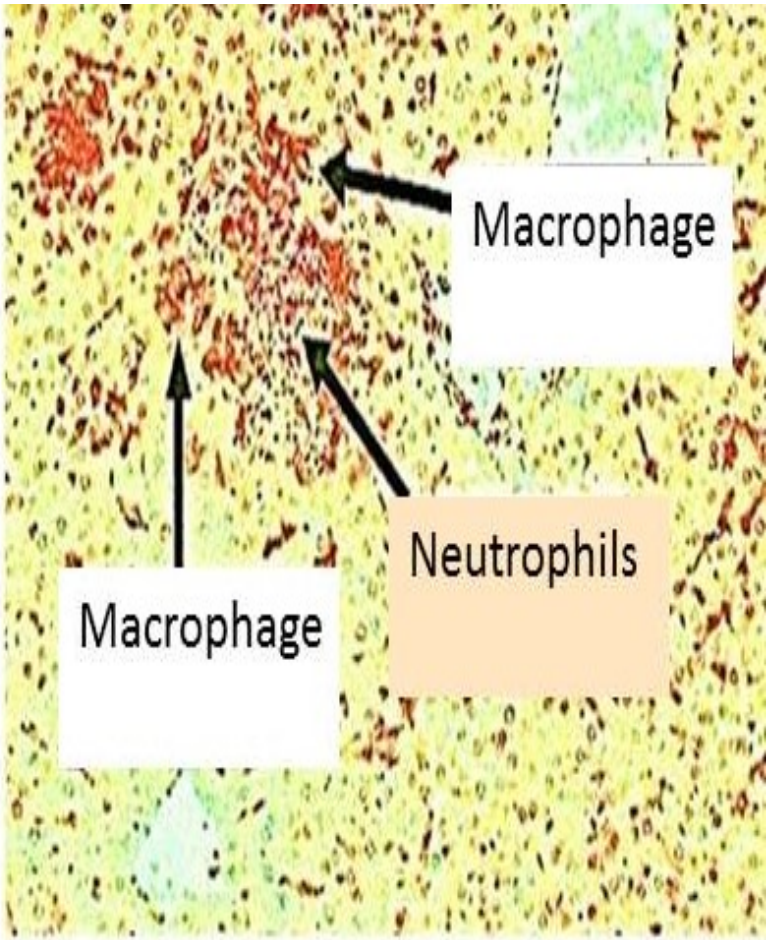
In these experiments we gauged the progression of disease in response to infection in the liver when the host is deficient in TCR γδ T-cells. We first designed a set of infection experiments in which we removed 10 infected WT and KO livers at days 1, 5, and 10 post-infection and compared the IHC and antibody staining results to each phenotype. In the infection models for each we observed differences that emerged in

FIGURE 3.3: MACROPHAGE INFLUX IN WT AND γδ T-CELL DEFICIENT LIVERS AFTER *S. TYPHIMURIUM* INFECTION

F4-80 anti-macrophage staining follows the macrophage influx and the neutrophil clearing that can be seen.5 dpi with $5.0x10^7$ CFU/ml. A) Macrophage staining in the infected WT shows no infiltration of macrophage at this stage in the infection but there is still the presence of neutrophils as shown in the blue hematoxylin stain. B) In infected γδ T-cell deficient liver macrophage cells shown highly concentrated at the site of infection; the blue hematoxylin counterstain shows infected KO tissue is still has a high concentration of neutrophils.



A) WT Liver , 5DPI, F4/80 anti-Macrophage clone, Vecta NovaRed detection and Hemotoxylin counterstain (20x)



B) KO Liver , 5DPI, F4/80 anti-Macrophage clone, Vecta NovaRed detection and Hemotoxylin counterstain (20x)

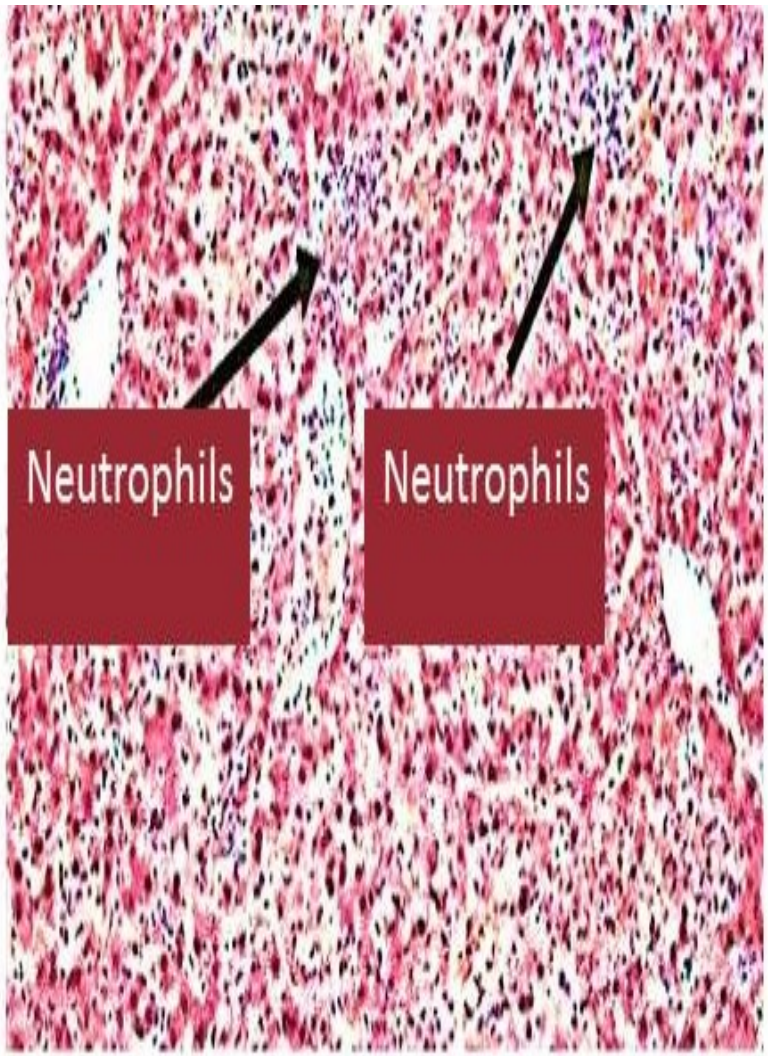
the progression of pathology at distinct times between the tissue types as seen in Figure 3.2. Structural changes emerge at 5 dpi in the KO that are not seen at this time in any of the WT tissue. The entire parenchyma in the KO is distinctively deformed as a large influx of neutrophils permeates the portal veins and portal ducts. The infiltration and clogging of the ducts and veins could be the cause of a secondary structural breakdown of tissue that is a consequence of an unregulated immune response to infection, rather than the due to infection only.

The WT also has a strong influx of the neutrophils during this point in time. However, their permeation of the tissue does not cause deformity in the tissue, particularly in the portal ducts and veins. It could be argued that there is an infiltration of the neutrophils in the KO based on the H&E stains alone of Figure 3.2b and 3.2c. While the KO also shows a degradation of vein and duct structure, these results could hint at the beginnings of tissue necrosis.

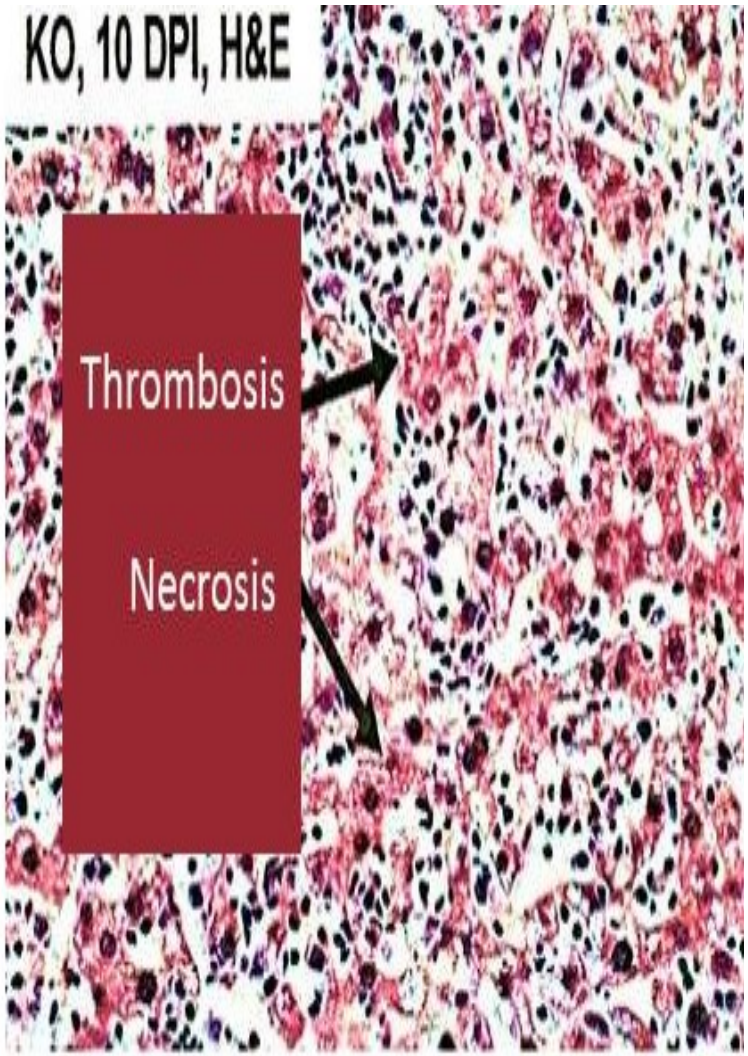
We examined the tissue with more sensitive immuno-staining. This staining targeted two rapid immune cells, which are the neutrophils and macrophages. In a normal infection, neutrophils are the first phagocytes to arrive at the location of infection, where they change their phenotype, become activated, and release cytotoxic antimicrobial molecules that include reactive oxygen species (ROS), oxidants, defensins, lactoferrin and cathelicidins.[115,116,117,118,119] They will also attract chemokines that, in turn, attract more neutrophils and monocytes. Moreover, in a properly function immune reaction these rapid immune responders are quickly cleared to prevent a prolonged attack on healthy tissue. On an important side note, this clearance makes the start of new tissue growth possible. Usually macrophages are the first of these immune cells that have a

FIGURE 3.4: TISSUE DESTRUCTION AS A RESULT OF IMMUNOPATHY IN THE LIVER

H&E stain of left lobule cross-section of liver infected with $5x10^7$CFU/ml of *S. typhimurium* at day 10 of the post-infection period (20x mag). A) In the WT there is a large influx of neutrophils in all the remnants of portal ducts and central veins; sclerosis of the parenchyma; and no signs of nascent vein or duct formation. B) In the KO liver there are no viable tissue visible; there is sclerosis to the extent that complete coalescing in parenchymal tissue; neutrophil in every area of liver section without macrophage clearance; no viable connective tissue or collagen structure; and visible fibrosis of blood vessels within the compartment.



A) WT Liver, 10 DPI, Hemotoxylin and Eosin Stain (20x).



B) KO Liver, 10 DPI, Hemotoxylin and Eosin Stain (20x).

clearance role, which includes clearing killed pathogen, necrotic tissue, and neutrophils and other white blood cells that stopped the initial pathogen attack.[120,121,122] It is understood that since we do not see substantial neutrophilic influx in the WT tissue at this stage of infection, we would not observe macrophage convergence at the site of neutrophil influx. In contrast, the neutrophils are still present in large numbers, and the "clearing" function of the macrophage has been hindered. This hindrance may be due the lack of regulation by γδ T-cell and the absence of signaling pathways that control macrophage functioning. Either scenario may be due to the lack of TCR γδ cells in the area of infection and, therefore, a lack of immune modulation for the infected tissue of the deficient phenotype.

As we have shown in Chapters 1 and 2, the lack TCR γδ T-cells in the gut renders that tissue compromised in immune-surveillance, tissue composition, and tissue function. This causes the tissue to be severely compromised when endangered with pathogen. The effector signals sent through the infected large intestine of the liver tissue can cause the γδ T-cell deficient liver to elevate its immune response more quickly than the normal liver. This may be why the neutrophil invasion was revealed earlier in the KO after pathogen invasion. The dysregulation by the absence of TCR γδ T-cells could be the reason for the lack of immune modulation for the infected tissue. It is understood that the influx of neutrophils is initially brought to this site through the mechanisms of a rapid host response. However, there is such an onslaught of neutrophilic bodies in the KO liver that there does not seem to be a regulatory halt to influx of these inflammatory proteins. Because of these findings it has been speculated that TCR γδ T-cells may have this regulatory function in modulating the inflammatory response that, when not controlled,

causes more tissue damage than the initial infection/injury.[123]

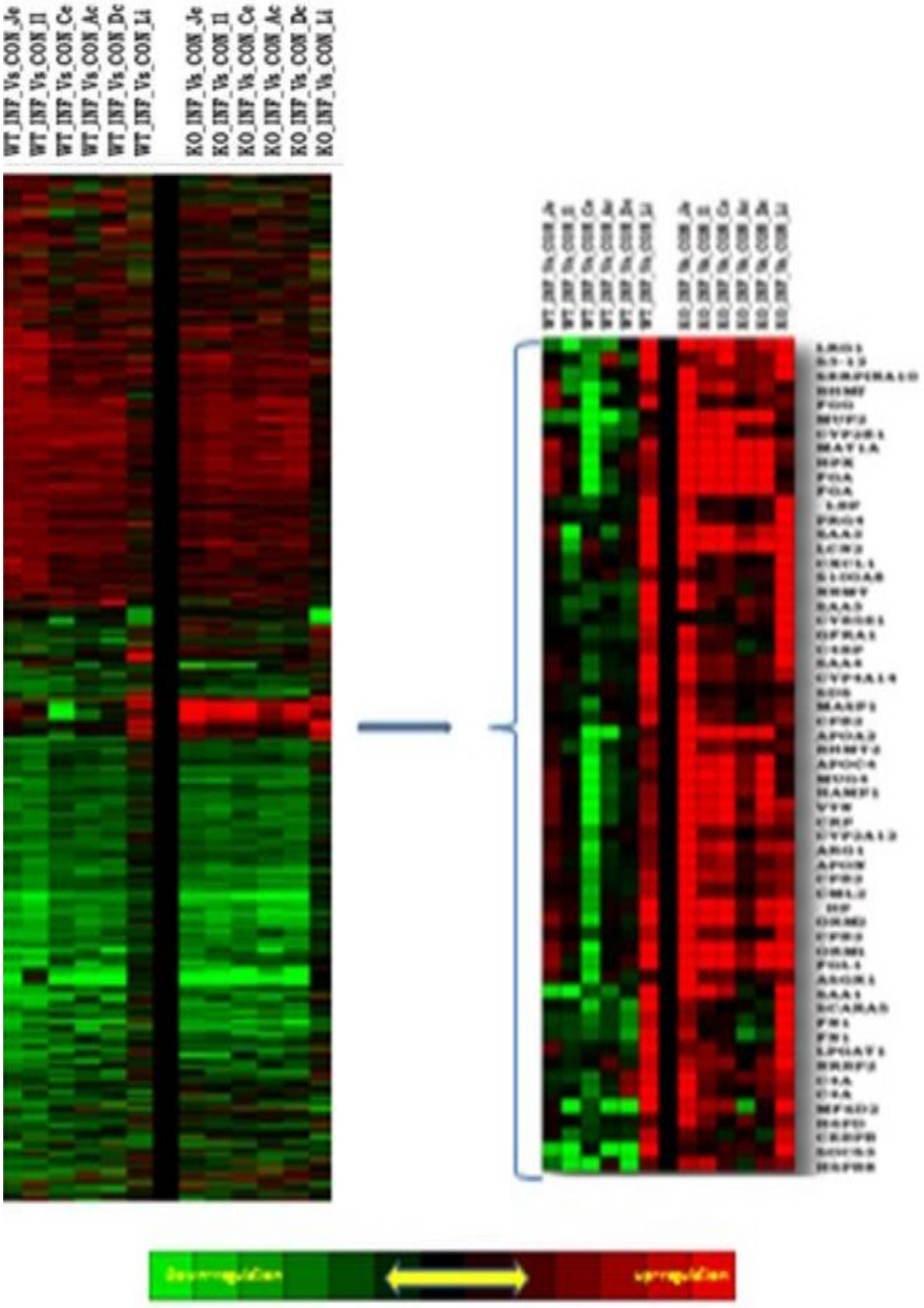
Transcriptome Analysis

Using the data and analysis from the IHC staining, we then preceded with whole genomic microarray of the liver. We hoped to compare the response of the two liver strains especially in infection. Second, we wanted to compare the responses seen in the liver to those seen in the GI. With the comparison to the genomic GI results, we are attempting to understand the timing of the liver immune response at the level of gene expression and at the level of phenotype expression that resulted in the phenotypic expression observed in the histopathology data. Using this microarray data would also help us to understand how the infection, which has its basis in the GI tract, affects the function of the neighboring liver tissue and explain the beginnings of a systemic breakdown in due to failure of the GI tissue lacking γδ T-cells.

At the time 16-hours post infection with *S. typhimurium*, the gene pathways most heavily up-regulated in the KO liver included those of the complement and coagulation cascades, cytochrome P450, and HDL pathways. The complement system is one of the oldest forms of immune response. It is not surprising, in light of the fact that the histopathology data reveals an neutrophil/macrophage infiltration as early as 5 dpi for the KO compared to the WT (who showed similar patterns later at 10 post infection). The complement and coagulation cascades continue the trend of infected KO lovers having the same genetic profiles in the heat maps generated in Figure 3.5 as the lower GI in the KO. While it is too early at 16 hours post infection for the *S. typhimurium* to have reached the liver of either strain (WT and KO) from the infected GI, there is still

FIGURE 3.5: GLOBAL GENE EXPRESSION IN WT AND γδ T-CELL DEFICIENT LIVER AS A RESPONSE TO WITH *S. TYPHIMURIUM* INFECTION

Heat map of global gene expression across all GI tract tissues and liver (the last tissue in both heat maps). Gene probes were normalized across samples an. Infected liver samples show a distinct genomic pattern that differentiates it from infected WT and KO GI tissues. There is a change in the genetic footprint in the liver of KO compared to WT in response to infection.


WT_INF_Vs_CON_Je
WT_INF_Vs_CON_Il
WT_INF_Vs_CON_Ce
WT_INF_Vs_CON_Ac
WT_INF_Vs_CON_Dc
WT_INF_Vs_CON_Li
KO_INF_Vs_CON_Je
KO_INF_Vs_CON_Il
KO_INF_Vs_CON_Ce
KO_INF_Vs_CON_Ac
KO_INF_Vs_CON_Dc
KO_INF_Vs_CON_Li
downregulation
upregulation

cell-to-cell communication and signaling of effector molecules. These signals may induce the deficient liver to ramp up the immune response earlier at the genetic level in anticipation of the infection.

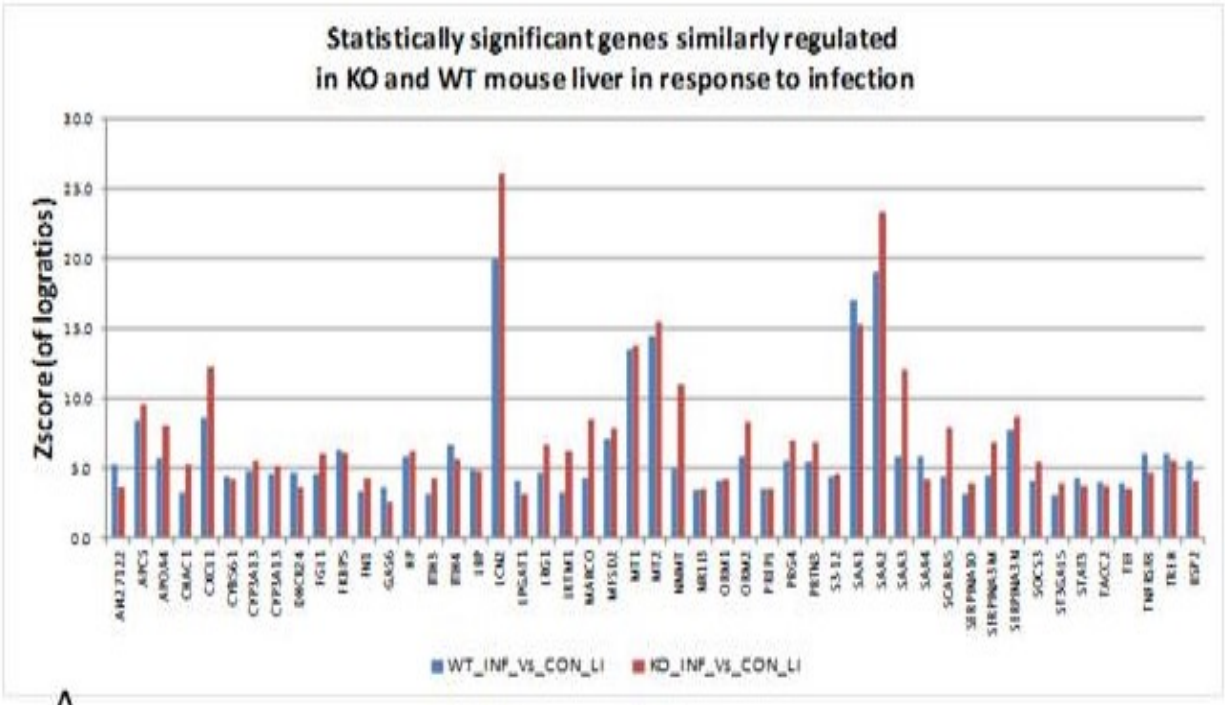
Many of the same genes are equally elevated in the infected livers of both WT and KO mice. The average overlap of DEGs for all the tissues of the GI tract was low for WT and KO compared to the overlap in the WT and KO livers (35% and 40%, respectively). The overlapping genes in liver specifically are enriched for the acute inflammatory response. This overlap may indicate a starting point of the immune process that begins away from the direct site of infection in which the regulation of the γδ T-cell is not a controlling factor in tissue response.

The significant overlap between WT and KO mice in terms of their transcriptional response to *Salmonella typhimurium* in liver could be the result of a reduced direct role for TCR γδ T-cells in liver in both WT and KO mice. This result could also be due to the fact that the infection itself starts in the upper GI tract and the actual *S. typhimurium* bacterium has not reached the liver tissue at 16 hours post infection. This means that at the transcription level, the response is due mostly to possible effector molecules and cell-to-cell communication between the infected tissue of the GI and infected liver. The greater significance of this is that on the transcriptional level, there is some correlation between the effects of infection found in the GI tract and the resulting responses seen in the liver. This indicates a failure of function in the GI due to the lack of γδ T-cell regulation and immune function has detrimental systemic failure.

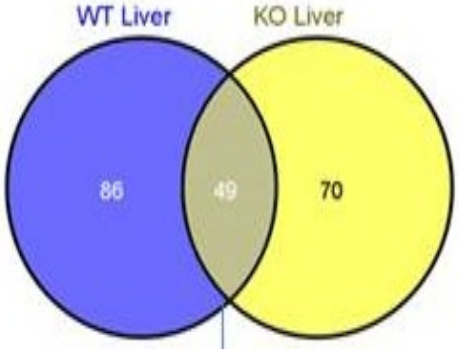
FIGURE 3.6: COMPARISON OF INFLAMMATORY RESPONSES TO *S. TYPHIMURIUM* INFECTION ON WT AND γδ T-CELL DEFICIENT LIVER

A. Statistically significant changes in gene expression (calculated as the Z-score of the difference between the means expressed as log ratios) for genes up-regulated in both WT and KO liver in response to $5x10^6$ CFU/ml infection of S. Typhimurium. Bar chart shows high concordance in response between WT and KO livers for these genes.

B. Venn diagram of DEGs of both WT and KO liver in response to infection. Genes for the acute inflammatory response show the greatest overlap.



A



B

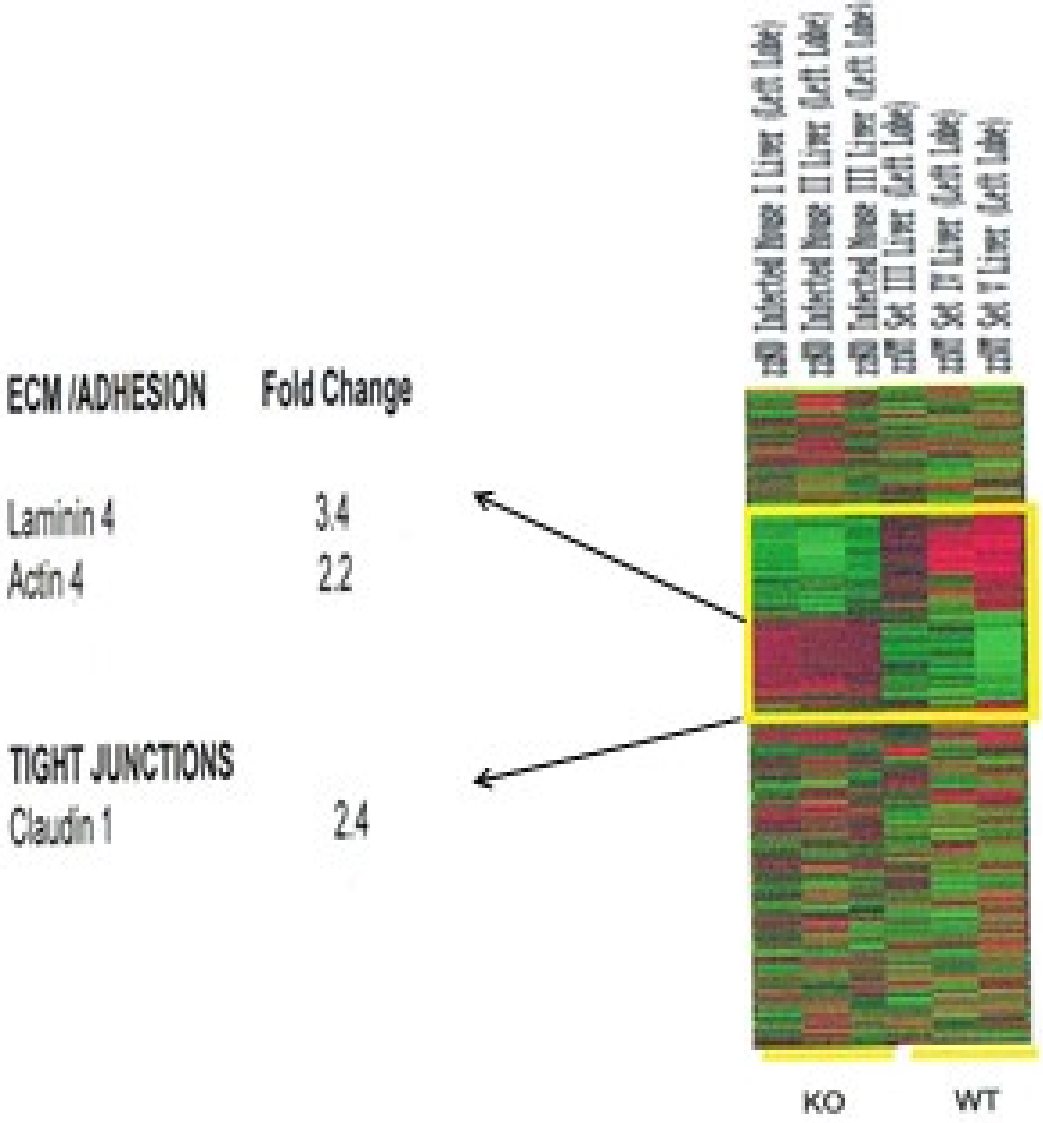
acute inflammatory response (p<8.5E-13), 10 genes: FN1, LBP, ORM1, ORM2, SERPINA3N, SAA1, SAA2, SAA3 , SAA4, STAT3

CONCLUSION

The liver is a peritoneal tissue that is distinct from the GI tissue that has been the main focus of this work. However, its proximity to the tissues of the GI do shed light on the: 1) response of γδ T-cell deficient and WT livers through effector molecules and the and the convergence of rapid response immune cells to the site near the site of infection once it has been introduced into the gut mucosa; 2) confirmation that tissue failure in the GI has a systemic effects on the first organs of contact such as the liver before it progresses throughout the host; and 3) progression of the pathogenesis of *S. typhimurium* infection once it has breached the gut mucosa and permeated bloodstream.

FIGURE 3.7: GENE EXPRESSION DIFFERENCES BETWEEN γδ T-CELL DEFICIENT AND WT LIVER DURING INFECTION

Heat map of genes showing the differential upregulation for KO liver in genes of the ECM/Adhesion genes Lamini4, Actn4, Cldn1 Genes show a fold change above 2.0 and p-values ≤ 0.05.


ECM /ADHESION
Fold Change
Laminin 4
3.4
Actin 4
2.2
TIGHT JUNCTIONS
Claudin 1
2.4
KO
WT

CONCLUSION

The gut epithelial functions to provide a protective barrier from enteric pathogens as well as to facilitate the absorption and distribution of needed nutrients. These functions are accomplished by the concerted action of a number of interacting cell types including epithelial cells and immune cells. Our hypothesis is that dysfunction of any one of these cell types will have adverse effect on the capacity of the epithelial tissue to continue their key functions.

Using multiple experiments, we studied the role of TCR γδ T-cells on the transcriptional, functional, and structural levels. It is clear from the microarray data presented here that the transcriptional program in γδ T-cell deficient mice has been altered across the multiple tissues of the GI tract before and after infection with *S. typhimurium* occurred. The functional and architectural changes observed in data from intestinal permeability and electron scanning data underscores the point that genetic differences seen earlier with the γδ T-cell deficient gut seem to be correlate with decrease of tissue functionality. The significance for our research is that these trends do show that the removal of the γδ T-cell from the gut environment renders intestinal epithelia vastly different in its genetic footprint and biological function.

In conclusion, we have demonstrated that the TCR γδ T-cells have a vital role in the proper functioning of the gut epithelia. TCR γδ T-cell helps support and maintain the gut epithelia and is the basis for a robust and effective response to enteric pathogen infection. The further study of these important γδ T-cell lymphocytes has broad implications for the examination of wound repair, therapeutic targeting for immune responses in the gut epithelia, and the understanding of the basic immune response to pathogen invasion in epithelial tissue.

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CURRICULUM VITAE for DESIRE' E. BARRETT
The Johns Hopkins University School of Medicine

DESIRE' E. BARRETT February 18, 2017

EDUCATIONAL HISTORY

Ph.D	2017	Program in Immunology Mentor: Mark J. Soloski, Ph.D	The Johns Hopkins University School of Medicine
M.S. (deferred)	2003	Biotechnology	The Johns Hopkins University Krieger School of Arts & Sciences
B.A.	1996	Biological Sciences	University of Maryland Baltimore County (UMBC)

PROFESSIONAL EXPERIENCE

October 2001-August 2004 SENIOR PROCESS DEVELOPMENT ASSOCIATE
SAIC-National Cancer Institute, Frederick, MD.
Developed protein/monoclonal antibody (mAb)/enzyme purification methods for projects in clinical trial-phase I. Designed and optimized western blots, ELISA and SDS-PAGE for biologic drug characterization and development. Wrote and edited cGMP-standard SOPs and batch record for regulatory compliance and BLA application. Isolated monoclonal antibodies (mAb) through protein precipitation, size-exclusion and affinity chromatography. Procured inventory adhering to

yearly budgets set by the NIH. Maintained effective communication with GMP Manufacturing, Formulation, Clinical, and QC for technology transfers. Supervised 3 drug product development projects from GLP-standard R&D into clinical trials as Liaison.

September 2000-September 2001 PROCESS DEVELOPMENT TECHNICIAN II
Cambrex (formerly Bioscience Contract Production Corporation, Inc.), Baltimore, MD
Led as Team Leader in the technology transfer of biologics from lab-scale to pilot production of bio-pharmaceuticals in clinical trial-phase I. Contributed to *F. tularensis* vaccine strain cultivation and purification, final-fill formulation, and lyophilization optimation in partnership with USARIID. Developed and maintained cell culture of *F. tularensis* live attenuated vaccine. Wrote and edited batch records and SOP for final fill and manufacturing protocols. Standardized notebooks for all personnel to meet GLP-standard and FDA regulatory standards.

January 1999-September 2000 MANUFACTURING PROCESS TECHNICIAN I
Cambrex (formerly Bioscience Contract Production Corporation, Inc.), Baltimore, MD.
Conducted recovery, isolation, and purification of recombinant protein enzymes and monoclonal antibodies (mAb) for the development of biologics and vaccines for clinical trial and BLA licensure. Recovered proteins and small molecules of interest

	through chemical lysis and fractionation of transfected cell culture. Chromatography column purification techniques include ion exchange, affinity, hydrophobic and gel exclusion. Quantitatively determined active pharmaceutical ingredient.
October 1998-December 1998	LABORATORY ASSISTANT Genvec, Inc., (Snelling, Inc. contract), Gaithersburg, MD. Cultured and amplified adenovirus for process development pilot-scale batch-fed fermentation. Adenovirus used as viral vector for gene-based therapies.
August 1998-October 1998	LABORATORY ASSISTANT Becton-Dickinson Microbiology Systems, (Kelly Inc. contract), Sparks, MD. Prepared ELISA-based diagnostic devices for research and development in immunology.

FELLOWSHIPS AND GRANTS

Sponsor:	National Institutes of Health
Identification number:	R21AI083555-01
Title:	Innate Immune Lymphocytes in the Gut Epithelium
Principal Investigator:	Mark J. Soloski, PhD.

Research support or the study of γδ T-cells in the Gut Designed and presented research that characterized the function of TCR γδ T-cells in normal and *S. typhimurium* infected GI epithelial tissue. Using high throughput microarray and proteomic analysis we discovered several novel γδ T-cell-induced genes and pathways that play critical roles in

controlling the immune response, homeostasis, and maintenance of the GI-Tract. Discovered potential therapeutic targets for translational research in complex disorders of the GI.

HONORS

March 2003	Letter of Commendation from the Majority Leader of the U.S. Senate William R. Frist (R., TN.) – Excellence in Extramural Team Research for Asparaginase Enzyme
May 2002	Team Leader, SAIC-National Cancer Institute, Protein Purification Project of L-Asparaginase from *Erwinia chrysanthemi*
September 2001	Team Leader, SAIC-National Cancer Institute, Protein Purification of Recombinant Saposin B Protein
February 2001	Liaison to GMP Process Scale-Up of Bio-pharmaceuticals

PUBLICATIONS

Barrett D. E., Cheadle, C. Berger, A.E., Watkins, T.[2], Barkataki, S., Boorgula, M., Barnes, K.C. and M. J. Soloski[1]. (2017) Global Transcription Analysis of Mouse Intestinal Epithelium reveals Critical Role of γδ T cells in Tissue Homeostatsis and in the Response to *S. typhimurium* Infection. (in preparation).

POSTERS AND ABSTRACT

D. Barrett, C. Cheadle, M. Soloski, A Genomic-Based Approach to Understanding TCR γδ T-Cells in the Gut Epithelial Compartment. American Association of Immunologists (AAI) Conference. Baltimore, MD, May 12, 2010.

D. Barrett, C. Cheadle, M. Soloski, A Genomic Approach in Studying TCR γδ T-Cells in the Intra-Epithelial Layer of the Gut Mucosa. Bayview Symposium. Baltimore, MD, March 22, 2008.

A. Kolhekar, S. Perry, **D. Barrett**, Purification Process Development of an Enzyme for Clinical Use Biopharmaceutical Development. National Cancer Institute Research Festival. Frederick, MD. May, 2004.

Abstract

A Genomic-Based Approach to Understanding TCR γδ T-Cells in the Gut Epithelial Compartment **D. Barrett**, C. Cheadle, M. Soloski, American Association of Immunologists Conference. Baltimore, MD. 2010

SERVICE AND LEADERSHIP

2008 - 2010 - Tutored doctoral candidates for the Graduate Oral Board Examination. Tutored and coached doctoral candidates in danger of losing their positions in their training program due to a "no-pass" initial grade on the board examination. Established study strategies and initiated mock examinations. All tutored candidates passed their re-examinations.

2006 Founding member of the Bio-Scholars Association of The Johns Hopkins University School of Medicine. There was an important need for minority PhD. candidates to have a group for outreach, mentorship, networking, specifically, on the Hopkins medical campus.